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A HABITAT TEMPLATE FOR *STUCKENIA PECTINATA*  
IN TE WAIHORA (LAKE ELLESMERE)

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A thesis submitted in partial fulfilment of the requirements for the Degree

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I would like dedicate this work to my parents (李 鐵珍, 胡 建科)  
and to my uncle (李 勝利)

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## Overall Summary

Te Waihora (Lake Ellesmere) is a shallow coastal lagoon located on the east coast of the South Island of New Zealand. An extensive macrophyte bed (primarily *Stuckenia pectinata* and *Ruppia spp.*) once existed around the lake margins. It disappeared after a storm event in 1968 and never returns. Now the lake is highly turbid and water column attenuates light rapidly through depth. The lake is periodically open to the sea, and salinity in the lake fluctuates as a result of the lake opening activities, and varies with distance to the lake opening. The aim of this study is to define a habitat template, a set of environmental factors (light attenuation, water depth, salinity) that allows *S. pectinata* to grow and persist indefinitely. A series of experiments were conducted to investigate: 1) the morphological and leaf photosynthetic physiological acclimation to low light conditions; 2) the leaf demography and leaf photosynthesis in direct and stepwise exposures to a gradient of salinity; 3) the interaction of light acclimation and salinity on leaf photosynthesis.

At low irradiance, primary shoots of *S. pectinata* grew taller, produced longer and lighter leaves and senesced leaves more quickly than at high irradiance. Salinity stress induced early leaf senescence and leaf production rate decreased with increasing salinity level. Plants did not tolerate salinity exceeding 20 ppt. Leaves at low irradiance had higher chlorophylls per fresh weight (per photosynthetic leaf area), suggesting enhanced light harvesting (capture of photon energy) efficiency per unit leaf area. Leaves at high irradiance had less chlorophyll per fresh weight but higher photosynthetic rate per unit chlorophyll, indicating higher light utilization (utilization of captured photon energy) efficiency. High salinity ( $\Rightarrow$  12 ppt) reduced photosynthetic rate per unit chlorophyll-a in leaves acclimated to high irradiance ( $340 \mu\text{mol}/\text{m}^2/\text{s}$ ) but not to lower irradiances ( $110$  and  $50 \mu\text{mol}/\text{m}^2/\text{s}$ ), suggesting salinity would reduce light utilization efficiency of surface reaching leaves but not submerged leaves in the turbid water column.

To conclude, turbidity and salinity synergistically limit the growth of *S. pectinata* in the lake: 1) both low-light stress in the water column and salinity reduce the number of leaves, therefore, less photosynthetic leaf area; 2) the photosynthetic rate per unit chlorophyll-a is either constrained by low-light stress in the water column or by salinity at or near the water

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surface. The knowledge of light and salinity limitation on growth of *S. pectinata* seems to well explain the dynamic of a population of *S. pectinata* in Te Waihora monitored over two consecutive growing seasons. In the end, a habitat template with defined ranges of water depth, light attenuation coefficient, and salinity was constructed for *S.pectinata* to grow in Te Waihora.

## Chapter 1: General Introduction

### Restoration of Macrophytes in Shallow Lake Ecosystem

Shallow lakes have the appearance of two states: one pristine state with crystal clear water and dense submerged macrophyte and the other disturbed state with turbid water of high concentrations of phytoplankton, cyanobacteria or (and) suspended solids (Scheffer et al. 1993). Each state has feedback mechanisms to re-enforce itself. The presence of submerged macrophytes reduces nutrient availability to phytoplankton, provides zooplankton refugia to enhance top-down trophic cascade of algae consumption, excretes allelopathic substances to suppress phytoplankton growth, and reduces sediment re-suspension (Figure 1) (Scheffer et al. 1994, Scheffer 1998). Those feedback mechanisms reduce turbidity and favour the growth of macrophytes. Whereas turbid water inhibits macrophyte growth and tends to exhibit high phytoplankton density and remain turbid (Scheffer 1998).

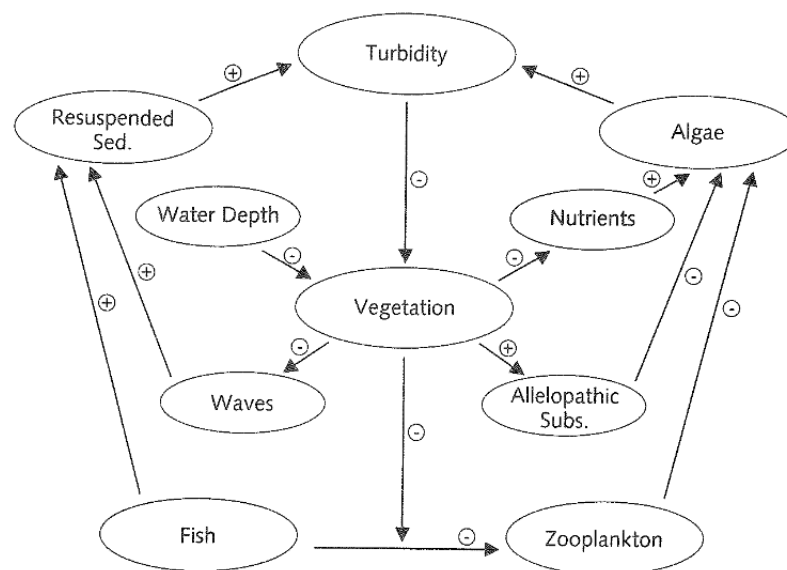


Figure 1. Diagram of self-reinforcing factors for clear water state and turbid water state (Scheffer 1998) (An arrow indicates an effect: a factor at the back of an arrow exerts an effect on a factor at the front of an arrow. Plus and minus sign beside an arrow indicates a positive effect and a negative effect respectively.)

Many shallow lakes in Europe, Australia, and New Zealand have undergone a shift from a clear water state into a turbid water state (Schallenberg and Sorrell 2009). A classic perspective is the shift could be driven by eutrophication, increase in nutrient input from

altered land use such as deforestation and agriculture. Submerged macrophytes and phytoplankton are both primary producers and compete for light and nutrient. In nutrient-poor lakes, submerged plants have the advantage to utilise nutrient in the sediment to out-compete phytoplankton, whereas in nutrient-rich lakes phytoplankton has the advantage to utilise sunlight and shades submerged macrophytes. The feedback mechanisms theoretically allow a clear water state to hold against increasing nutrient level to a threshold, above which shallow lakes shift into the turbid state (Scheffer et al. 1993). Probable factors triggering the shift to a turbid state are increased growth of phytoplankton and periphyton (because of increased nutrient concentrations), and direct damage to vegetation by fish and birds, storms, herbicides and water table rise (Scheffer 1998).

Shifts into turbid water state reduce biodiversity, threaten drinking water supply, and are aesthetically unpleasant (W. et al. 2016). Substantial efforts have been devoted to restorations of turbid lakes into a clear water state. Successfully restored lakes always have long-term stable submerged macrophyte communities (Hilt et al. 2018). Theoretically, only reduction in nutrient to below a critical threshold level or removal planktivorous and benthivorous fish leads to a recovery of clear water state and return of submerged macrophytes (Scheffer et al. 1993). External nutrient reductions to restore submerged macrophyte communities are not always successful, with many lakes characterised by a clear state in spring and turbid state in late summer (Hilt et al. 2018). Other in-lake measures (bio-manipulation or sediment removal) result in transient clear state in both spring and summer that reveals to turbid state after a few years (Hilt et al. 2018). Stable clear water state associated with dense macrophytes beds sometimes come decades after reduction of external nutrient loadings (Scheffer 1998, Hilt et al. 2006), or when in-lake measures are combined (Hilt et al. 2018).

### **Macrophytes in Te Waihora (Lake Ellesmere)**

Te Waihora (Lake Ellesmere) is a large (198 km<sup>2</sup>), shallow (mean depth = 1.4 m) and brackish lagoon located south of Christchurch, New Zealand. In Te Waihora (Lake Ellesmere), macrophyte beds existed around the margin of the lake and were comprised mainly of *Ruppia megacarpa* and *Stuckenia pectinata* (Gerbeaux 1989). The macrophyte bed declined

in the 1920s and the rate of decline became more rapid until its disappearance in the 1940s (Mason, 1946). Significant recovery happened in the 1950s and significant growth (Figure 2) continued until the “Wahine Storm” on the 10th April 1968. The storm removed the macrophyte beds. Afterwards, macrophytes only occur occasionally in small patches in the lake (Gerbeaux 1989, Jellyman et al. 2009). Intensive agricultural activities have increased since 1970 in the catchment area (Lomax et al. 2015) and Te Waihora (Lake Ellesmere) is now hypertrophic and in a turbid state. It is questionable whether submerged macrophyte would have returned if there had been no increase in agricultural activities in its catchment area. However, the disappearance of an extensive submerged macrophyte bed over decades after the storm is likely attributed to eutrophication, as introduced in the previous section.

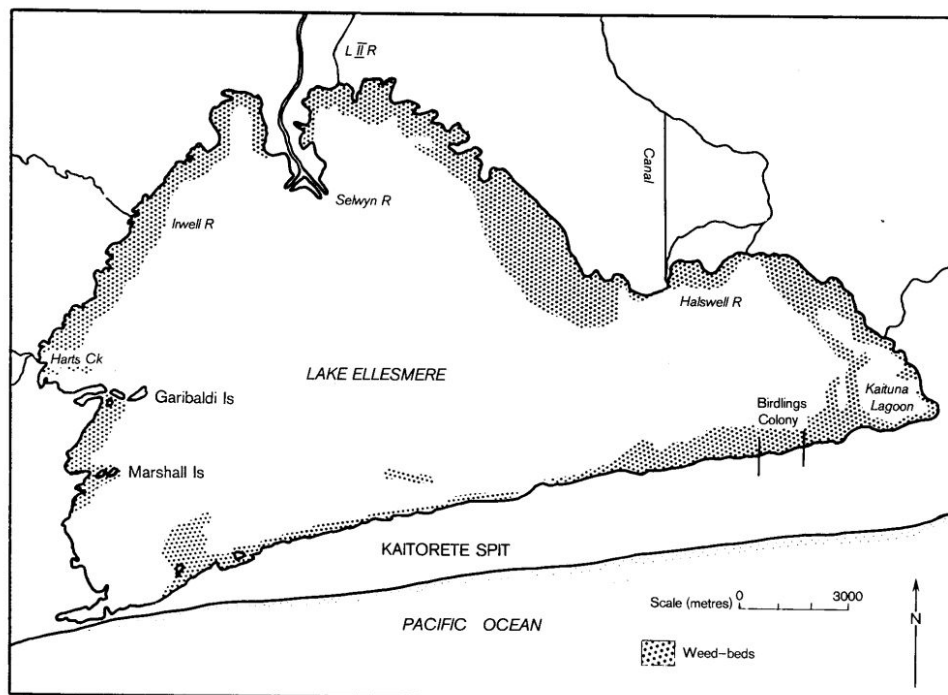


Figure 2. The distribution of submerged macrophytes in Te Waihora in early 1960s (from Hughes et al. 1974)

As a hypertrophic lake, the lake water has high concentrations of phytoplankton and suspended solids due to nutrient enrichment and strong wave actions. Scattering by inorganic particles is the main cause for light attenuation through the water column (Gerbeaux and Ward 1991), therefore, the lake water is highly turbid (close to 200 NTU). Turbidity is a measure of light scattering by the particles in the water. Re-suspended

sediment contributes 80% to the light attenuation through the water column and phytoplankton contributes another 20% (Hawes and Ward 1996). Despite highly turbid, moderate to high levels of phytoplankton biomass are somehow supported (Gerbeaux and Ward 1991). Strong vertical mixing induced by wave actions perhaps circulates phytoplankton to or near the water surface where light is sufficient for growth (Gerbeaux and Ward 1991). In Te Waihora (Lake Ellesmere), the compensation depth for net photosynthesis, the depth at which algal photosynthesis balance respiration, was ranging from less than 0.1 m in winter to 0.3 m in summer (Hawes and Ward 1996). Larned and Schallenberg (2006) estimated the depth to which 1% of surface light can penetrate, often taken as the limit to macrophyte growth, to be around 0.4 m based on data of Secchi depth from 1987 until 2005. Light availability is concluded to be the primary constraint on the growth of submerged macrophytes in Te Waihora (Gerbeaux 1993).

In addition to the poor light condition in the water column, strong wave actions and salinity also stress the growth of submerged macrophytes in Te Waihora (Lake Ellesmere). Wave action disturbs the establishment of submerged macrophytes and determines the minimum colonisation depth (Chambers 1987, Hawes et al. 2003). The minimum colonisation depth was estimated to be 2.1 m (Jellyman et al. 2009), deeper than the estimated light compensation depth of 0.3 m (Hawes and Ward 1996) .

To prevent flooding the land adjacent to the lake, Te Waihora (Lake Ellesmere) is regularly open to the Pacific Ocean via digging a channel at the southwest corner of the lake (Figure 2). The lake is connected to the sea when water level reaches 1.05 m above mean sea level from September to April, or 1.14 m from May to August (North Canterbury Catchment Board). The lake is naturally closed after opening and lake openings lasted 25 days on average in 1978-1980 (Lineham 1983), and the opening of 20 - 30 days is estimated to raise the lake salinity level by 1.5 to 4 ppt (Gerbeaux 1989). The opening longer than 30 days likely raises the salinity level by 10 ppt or more, at least for the southwest half of the lake (Gerbeaux 1989). Periodic seawater intrusion renders the lake saline with salinity fluctuating between 5 and 10 ppt most of the time (Gerbeaux 1989). Spatially variations in salinity in Te Waihora (Lake Ellesmere) form a gradient towards the lake opening (Figure 3).

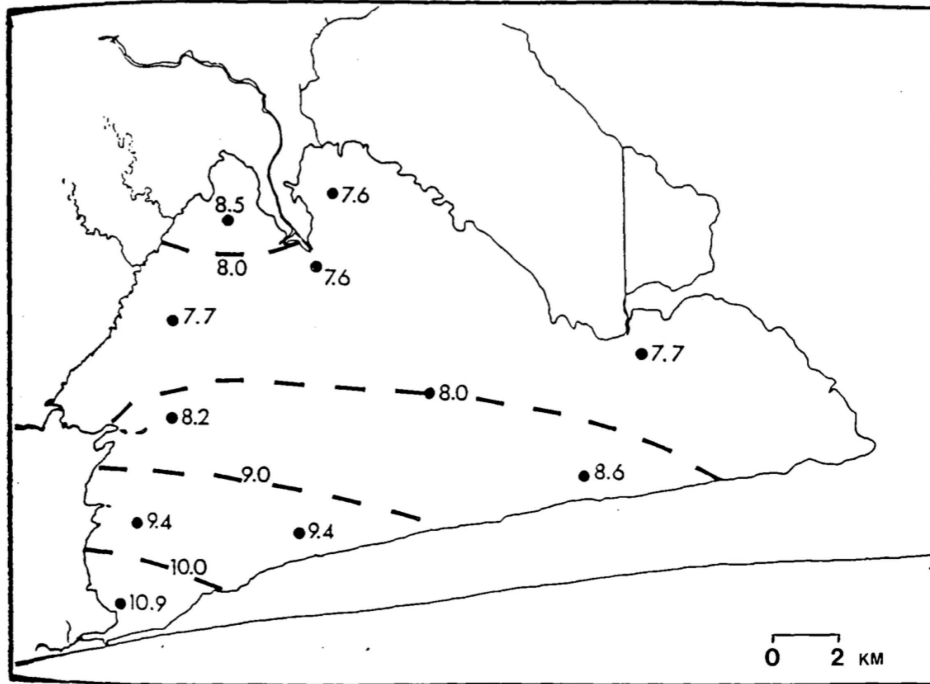


Figure 3. Mean salinity (in ppt) in Te Waihora (Lake Ellesmere), 1978 - 1980, from Lineham (1983)

Salinity fluctuations associated with changes in the lake opening regime may well explain the pattern of decline and recovery from the 1920s to the 1960s (Gerbeaux 1993). Long-period lake openings in the 1930s and 1940s caused raised salinity levels and might relate to the recession of the macrophyte beds. Whereas more frequent and short period lake openings in the 1950s kept the salinity low and less volatile, and encouraged the recovery of the macrophyte beds (Gerbeaux 1993).

At a sheltered site (Timber Yard point) at the western shoreline of the lake (west to the Garibaldi Island in Figure 2; also Overton's bay in Gerbeaux 1989), a small population of *S. pectinata* was found in 1986-1987 (Gerbeaux 1989) and in late summer in 2015, and spring in 2016 and 2017. The monthly salinity at this site (Timber Yard Point) ranges from 2 ppt to 27 ppt (Figure 4), with an average of 8.4 ppt and a standard deviation of 3.8 ppt (data from Environment Canterbury).

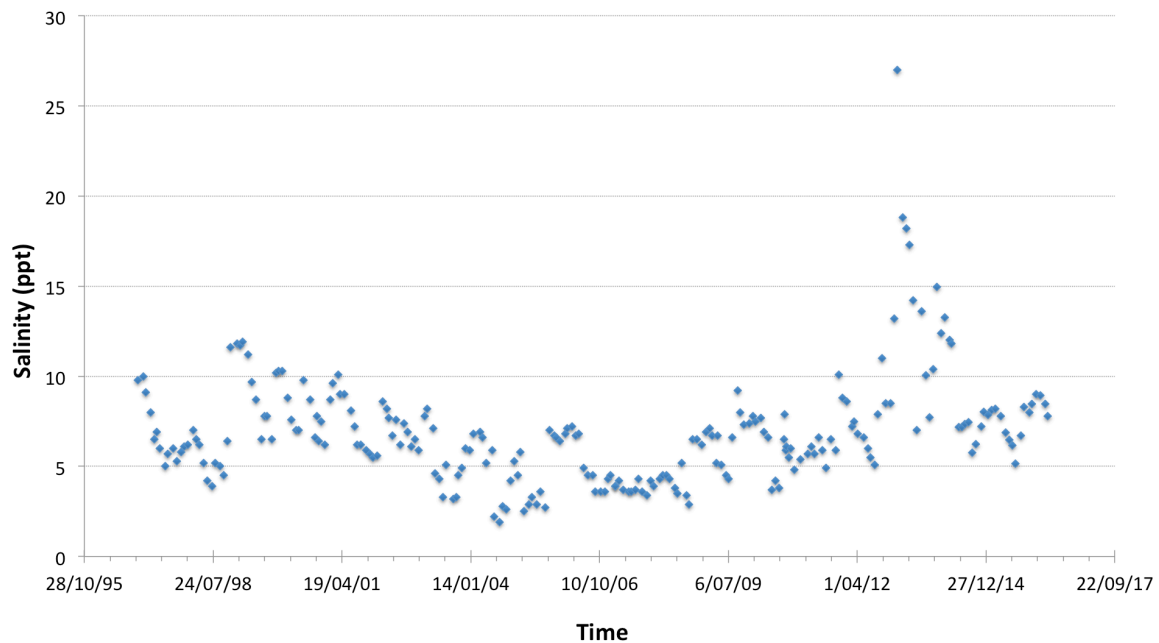


Figure 4. Monthly salinity at the Timber Yard Point from 1995 to 2017 (data from Environment Canterbury)

To rejuvenate the ecosystem health of Te Waihora (Lake Ellesmere), Ngai Tahu, Environment Canterbury and the Ministry for Environment collaboratively launched the Whakaora Te Waihora programme. A goal of the programme is to re-establish an indigenous macrophyte beds over 20 years and to recover 50% of the marginal macrophyte beds in the lake over 50 years (<http://tewaihora.org/the-plan-2/>). The macrophyte beds are expected to reduce wind-driven sediment suspensions, to compete with phytoplankton for nutrient and light in the water, therefore to improve water clarity. In Te Waihora (Lake Ellesmere), macrophyte beds are also expected to enhance biodiversity, to buffer nutrient cycling from input waterways and to increase lake productivity (Gerbeaux 1989). In addition, a clear water state of Te Waihora (Lake Ellesmere) associated with dense macrophyte beds in the past is connected with the spiritual values of local residents, such as the Tikanga Māori and mahinga kai. The ecological and spiritual values of a clear water state of Te Waihora are regarded as the most outstanding merits in the National Water Conservation (Lake Ellesmere/Te Waihora) Order of 1990. The re-establishment of the indigenous macrophyte bed in Lake Te Waihora (Lake Ellesmere) is thus important from both ecological and cultural points of view. The hypothesized constraints on growth of macrophyte in Te Waihora (Lake Ellesmere) are low light conditions, salinity fluctuations (Gerbeaux 1989), and wave actions (Jellyman et al. 2009).



## ***Stuckenia pectinata***

*Stuckenia pectinata* was a primary species forming the submerged macrophyte bed around the lake marginal area (Gerbeaux 1989), and one population still occurs at a sheltered site in the lake (field work). *Stuckenia pectinata* is considered a suitable indigenous species for restoration due to its tolerance to turbidity (Gerbeaux and Ward 1991) and to fluctuating salinity (Jellyman et al. 2009). The characteristics of the plant are introduced below.

### ***Morphology***

*S. pectinata* (previously named *Potamogeton pectinatus*) is a nearly cosmopolitan submerged angiosperm (Kantrud 1990). Morphologically, it is a tall aquatic plant rooted in sediment, perennially submerged except for inflorescences, and has long stems and small, mostly undivided, linear and non-floating leaves (Hutchinson 1975). The plant generally has an extensive subterranean system of rhizomes -- a robust rooting system (van Wijk 1988). The roots (about 0.2 mm diameter) penetrate the bottom to at least 0.5 m in sand, but to a much shallower depth and are proportionally smaller in finer sediments (Kantrud 1990). Rhizomes are of 1 to 5 mm in diameter and rhizomes of 5 mm in diameter have been observed in robust plants living in sediment with a thick organic layer (van Wijk 1988). Rhizomes have bare nodes alternating with nodes from which a shoot and a root develop (van Wijk 1988). Rhizomes first emerge from the third or fourth node of the main stem during tuber sprouting. In the later stages of growth, rhizomes also develop from the main stems (Kantrud 1990).

*S. pectinata* usually appears in monotypic stands. Secondary stems can grow from rhizomes emerging from the main stem and reach the water surface several weeks after the main stem. Main stems have longer shoots with thicker leaves and less branchings, while secondary stems have profuse branchings (Kantrud 1990). Stems vary considerably in length (up to 2 m). Some populations produce long stems with the branching concentrated in the upper part of the shoot, forming densely-leaved “brushes” at the water surface. Other populations produce shorter shoots with the branching along the shoots (van Wijk 1988). Also, the density of the shoots varies among populations. The leaves range from 50 to 180 mm in length and 0.25 to 2.5 mm in width (van Wijk 1988).

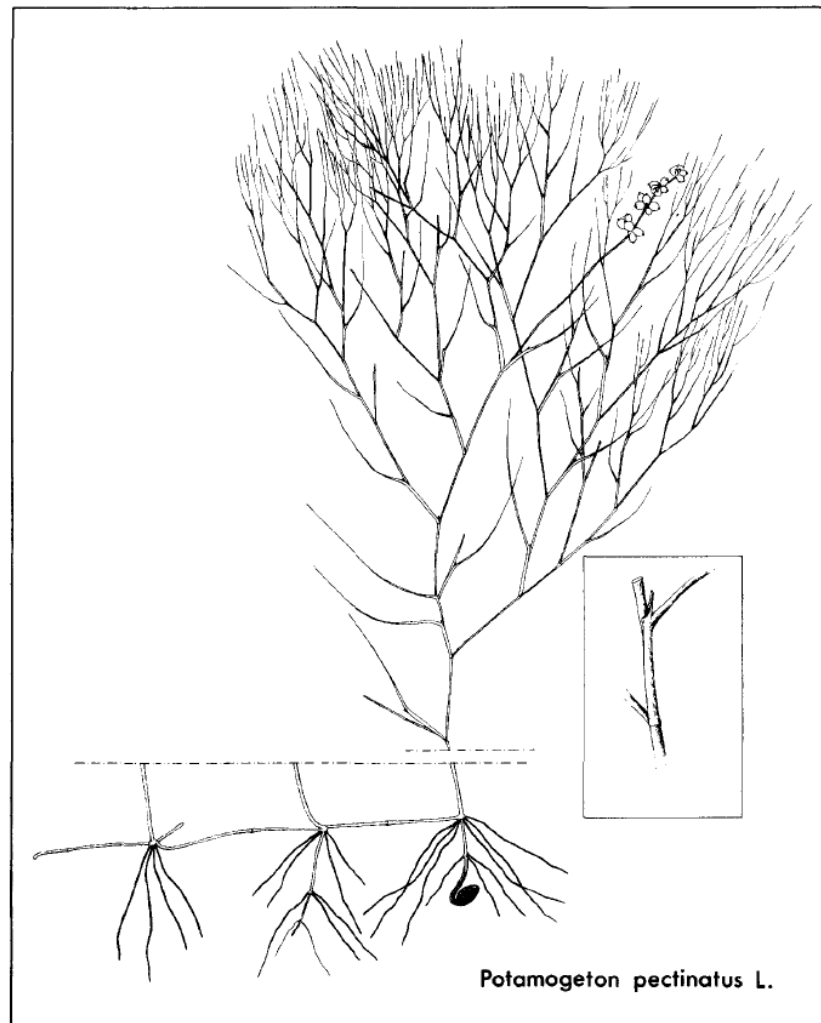


Figure 1. General habit of *Stuckenia pectinata* (previously named *Potamogeton pectinatus* L.), showing ripe achenes and the tuber from which the plant developed. Inset: characteristic leaf-sheath with ligule. From van Wijk (1988)

## Life Cycle

In the northern hemisphere, various hibernating organs of *S. pectinata* begin to grow when water temperature reaches 10 °C (late March – late June). Plants reach the water surface in May to mid-July and healthy stands occupy much of the water surface around 2 weeks later (Kantrud 1990). Senescence of vegetation starts from late August to October when water and sediment temperature drops (Kantrud 1990). Flowering usually happens during mid-May to mid-July after the maximum biomass of vegetation is attained. From mid-June to mid-August, water-derived pollination occurs and achene growth starts (Kantrud 1990).

Different populations of *S. pectinata* appear to have either an “annual life cycle” or a “perennial life cycle”. The “annual” or “perennial” life-cycle reflects how well the habitat favours their growth. A “perennial” life cycle population was found in sheltered and oligohaline waters, whereas four other “annual” cycle populations were found in either open saline water or shallow water with strong winds and grazing by waterfowl (van Wijk 1989a). In favourable habitats, plants can survive in winter as intact plants, although aboveground biomass is significantly less than in summer. Shoots, rhizomes, roots, tubers, and achenes can all contribute to the recovery of the population in the next growing season. The alternative life cycle occurs in less favourable habitats, where the aboveground and belowground parts of the plants die completely in autumn. Tubers and achenes are the only organs after winter and are responsible for new plants establishment in the next growing season (Table 1).

Table 1: Comparison of characteristics of populations with different life-cycle (summarized from populations studied in van Wijk (1988))

Life cycle	Biomass yield	Tissue surviving winter	Growth period	Size of tuber	Stage of tuber production
“Perennial”	High	Tubers, shoots, rhizomes, roots	long	Large and heavy	Later in growing period
“Annual”	Low, particularly underground biomass	Only tubers	short	Small and light	Early in growing period

### ***Reproduction and the significance of tubers***

Achenes are the sexual reproduction organs of *S. pectinata*. Seed bank formation depends largely on the degree of shelter of habitats (van Wijk 1989a). Persistent seed banks, however, only make a limited contribution to the short-term survival of populations because the germination of achene is proved to be poor. Usually, plants derived from achenes are uncommon in permanent wetlands and sexual reproduction by achene may function as a long-term survival strategy, such as the re-establishment of a population after long-term desiccation or drastic changes in salinity (van Wijk 1988, Kantrud 1990). The achenes are probably eaten and dispersed by waterfowl (van Wijk 1989a). Generally, low or absent genetic variation within populations indicates the significant role of asexual reproduction

(van Wijk et al. 1988) and vegetative reproduction appears to be the dominant reproduction strategy for *S. pectinata*. When a growing season begins, shoots, apical shoots and rhizomes start to regrow, and even part of detached shoots washed ashore may be able to develop into a new plant (van Wijk et al. 1988).

Tubers are the most important vegetative reproduction tissue. Production of tubers begins when main stems develop a horizontal rhizome near the surface of the sediment. The rhizome then penetrates into the sediment and forms branches at every other node, and tips of these branches can develop into tubers (Kantrud 1990). Tubers are formed in most of the population and are the only reproductive structure in “annual” populations (van Wijk et al. 1988, Kantrud 1990).

Tuber formation in autumn is initiated by short photoperiods (Spencer and Anderson 1987). van Vierssen and Hootsmans (1994) further showed that short photosynthetic periods (cumulative periods in a day when the net photosynthetic rate is positive) are responsible for tuber initialization and the photoperiod did not trigger tuber initiation. Tuber formation probably competes for resources with the growth and maintenance of old tissues, and may lead to early senescence of plants when energy from aboveground biomass is allocated to tuber production (van Vierssen et al. 1994).

Heavier tubers produce large plants that reach the water surface earlier and develop more shoots, whereas smaller tubers produce plants at a reduced growth rate (Spencer 1987). Smaller tuber size was speculated to be an adaptation to fewer photosynthetic tissues (aboveground biomass) and a shorter growing period (Pilon et al. 2003). New tubers were ready for germination upon detachment from the rhizome and it is the rhizome that forces tuber dormancy (van Vierssen et al. 1994). Tuber germination is enhanced by exposure to a cold temperature through winter. If the period of exposure to cold is long enough, tuber germination becomes insensitive to light and salinity (van Wijk 1989a). In insufficient light conditions, new plants can rely on their tubers for about 14 days (van Vierssen et al. 1994).

## **Habitat Templates of *Stuckenia pectinata* in Te Waihora (Lake Ellesmere)**

To recover a submerged macrophyte bed of *Stuckenia pectinata* in Te Waihora, empirical experience of restoration of shallow eutrophicated lakes into a clear water state may not provide enough guides, because Te Waihora has fluctuating salinity and strong wave actions besides turbidity. The aim of this study is to develop a habitat template that describes a set of key environmental factors in the lake, each with a defined range. Within the habitat template, *Stuckenia pectinata* in Te Waihora (Lake Ellesmere) is able to grow with appropriate acclimations (e.g. etiolation and shade adaption to low light), and to persist indefinitely. To screen for the key environmental factors to construct the habitat template, a successful restoration story of a submerged macrophyte bed of *S. pectinata* in a shallow lake is reviewed, and a few potentially limiting factors for the survival of *Stuckenia pectinata* are reviewed. Jellyman et al. (2009) recommended utilisation of artificial wave barriers to assist macrophyte regeneration in Te Waihora, therefore wave action is not considered in the habitat template.

## **Restoration of *Stuckenia pectinata* in Lake Veluwe**

Lake Veluwe (40 km<sup>2</sup>, mean depth 1.3 m) is a shallow, artificial and eutrophic lake in the Netherlands. This lake represents many shallow lakes that have been through the process of eutrophication in the country. At the end of the 1960s, the lake was occupied by dense mono-specific stands of *Stuckenia pectinata*. In the early 1970s, eutrophication reduced ecosystem diversity, deteriorated water quality, and limited the coverage of *S. pectinata* in the lake to only 5% in 1975. The lake seston was then dominated by the cyanobacterium *Osillatoria agardhii* Gomont (Scheffer et al. 1994). In 1980, a restoration project, involving a reduction in phosphorus loading and flushing of the lake, resulted in a drastic reduction of the contribution of algae to light attenuation. *S. pectinata* recovered as transparency of the lake water increased (Figure 2). The main factor that explains the decline and recovery of *S. pectinata* in Lake Veluwe is concluded to be light. In addition, the development of periphyton is regarded as an important factor triggering the decline of *S. pectinata* in Lake Veluwe. In the lake, periphyton coating on macrophytes is reported to attenuate 20 -30% of incident light within two weeks and up to 65% during spring blooms (Vermaat 1994).

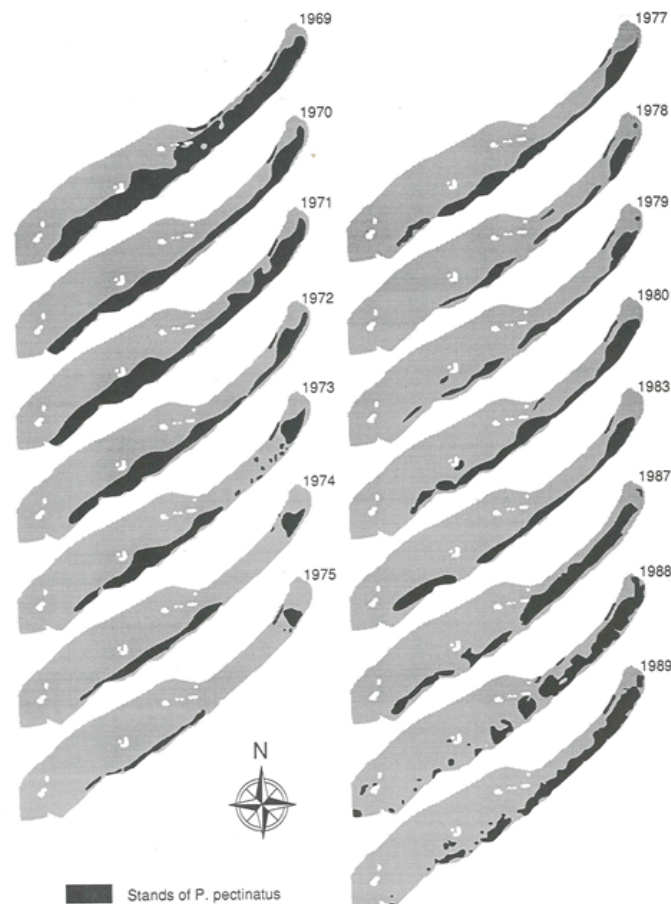


Figure 2. Distribution of *Stuckenia pectinata* in Lake Veluwe from 1969 to 1989. From Scheffer et al. (1994)

## Key Environmental Factors

### Light

Light quantity, specifically photosynthetically active radiation (PAR), determines the maximum depth that macrophytes can colonize (Spence 1982). Light is a principal factor controlling photosynthesis and the growth of macrophytes (Wetzel and Neckles 1986). Light quantity at a given depth is determined by the incident irradiance, the depth, and the light attenuation coefficient of the water. Light attenuation is determined by the scattering and absorption of light through the water column. In Te Waihora (Lake Ellesmere), light scattering by particulate matter and plankton makes the water highly turbid. High turbidity, therefore strong light attenuation, creates low light quantity in the water column, unfavourable for the growth *S. pectinata*.

*S. pectinata* is both morphologically and physiologically flexible in acclimation to different light conditions. Morphological acclimations to a rapid attenuation of light often involve a concentration of photosynthetic tissue close to the water surface (Kantrud 1990). Plants cultured at low irradiance have fewer shoots, enhanced vertical shoot extension through increased internode length, and increased stem biomass compared to plants at high irradiance (Hootsmans et al. 1996, Pilon and Santamaria 2002a). In addition, Kantrud (1990) also reported that insufficient light can make *S. pectinata* have fewer and coarser leaves, loss of branching and basal decay. Plants grown at higher light intensity demonstrate less elongation, produced more leaves and formed more secondary shoots than those grown in shade (van Vierssen and Hootsmans 1994). Physiologically, chlorophyll concentration of the leaf is increased in low light irradiance (Hootsmans et al. 1996, Pilon and Santamaria 2002a). These acclimations allowed a similar photosynthesis and biomass accrual rate to occur at low irradiance level as compared to high irradiance (Hootsmans et al. 1996, Pilon and Santamaria 2002a). These acclimations are genetically determined as Pilon et al. (2003) found similar photoacclimation responses in *S. pectinata* clones collected from different habitats.

Photoacclimation responses also interact with photoperiod and time. Shorter photoperiod increased leaf to plant biomass ratio and maximum photosynthesis (Pilon and Santamaria 2002a). Increased age of the plants decreased leaf to shoot biomass ratios, maximum photosynthesis, leaf chlorophylls content, and respiration (Hootsmans et al. 1996).

## Temperature

In Te Waihora (Lake Ellesmere), temperature ranges from 6.5 °C to 21.5 °C (Lineham 1983). Temperature has a regulatory effect on growth, phenology and resource allocation to propagules in *S. pectinata* (Kantrud 1990). The optimum growth of *S. pectinata* occurs from 23 to 30 °C in the laboratory (Spencer 1986) and 25 to 28 °C in a Wisconsin stream (Madsen 1986). 5 °C is considered the lower limit for growth (Hodgson and Otto 1963). *S. pectinata* died at 38 °C in a culture experiment and stopped growing at 37 °C in another experiment (Kantrud 1990).

With increasing temperature, *S. pectinata* shows higher growth rates and higher maxima for the number of leaves, leaf bundles and secondary roots (Kantrud 1990). Pilon and Santamaria (2002b) reported *S. pectinata* increased the number of nodes per shoot, the total number of shoots, total leaf area per plant, and below-ground biomass to overall biomass ratio with increasing temperature (up to 25 °C), while stem biomass to overall biomass ratio decreased with increasing temperature. The final biomass yield increased between 10 and 15 °C and levelled off at higher temperatures (20-25 °C) (Pilon and Santamaria 2002b).

Interestingly, optimum temperatures for both net and gross photosynthesis of *S. pectinata* do not depend on the growth temperatures, and the highest gross maximum photosynthesis is found in plants grown in 20 °C (Pilon and Santamaria 2002b). Respiration rate increases between 10 and 20 °C and levels off or even decreases at higher temperatures (Pilon and Santamaria 2002b). Tubers can germinate and grow at 5.5 °C, though 25 °C is the optimum for tuber germination (van Wijk 1983). For germinating tubers, temperature more than light affects the rate of shoot elongation, leaf production and the development of important pigments, because they can rely on carbohydrate from tubers (Spencer 1986). The temperature during the period of tuber germination and the period of early growth can affect the ultimate peak biomass of the plant, and subsequent resource allocation to tubers. Low water temperature results in lower overall plant biomass and more biomass allocation to tubers, while high water temperature does the opposite (Kantrud 1990).

## Salinity

In thalassic waters, optimum salinity for *S. pectinata* is from 5 to 14 ppt. In waters with Cl<sup>-</sup> as dominant anion the optimum salinity for *S. pectinata* is 3 to 6 ppt (Kantrud 1990). Salinity at 3 ppt stimulated while more than 6 ppt prevented tuber growth, and biomass production decreased with increasing salinity above 3 ppt (Teeter 1965). Lumsden et al. (1963) found a positive relationship between tuber production and salinity (between 0.6 to 5.4 ppt). It is still unknown how *S. pectinata* tolerates salinity. van Wijk et al. (1988) suggested optimum salinities for *S. pectinata* are partially genetically determined. Generally, *S. pectinata* grow



best in terms of the number of shoots and biomass production in low salinities similar to its original habitat (Kantrud 1990). Salinity tolerance is an advantage for *S. pectinata* in competing with other species.

## Nutrient

Kantrud (1990) concluded that *S. pectinata* is unlikely to be limited by the amount of nitrogen (N) in natural waters because of its ability to use sediment N. Concentrations of N in plant tissue can be 10-18 times of sediment N, and 1000 to 1500 times of water column N (Gopal and Kulshreshtha 1980). The plants likely prioritize sediment N over water column N, as substantiated by  $\text{NH}_3$  depletion in sediment supporting dense *S. pectinata* (Kantrud 1990). Phosphorus (P) can be absorbed by *S. pectinata* from water via shoots and sediment via roots (van Wijk 1989b). More likely, sediment P supports *S. pectinata* in natural waters. van Wijk (1983) showed a lake supporting *S. pectinata* with a perennial life cycle had water column  $\text{PO}_4^{3-}\text{-P}$  less than 0.2 mg/L, but high sediment P. Howard-Williams and Allanson (1981) showed that the upper 5 cm of sediment was a major source of P for dense *S. pectinata* (over 1,000 shoot per square meter) in a large wetland, while water column  $\text{PO}_4^{3-}\text{-P}$  was less than 5  $\mu\text{g/L}$ . Van Vierssen and Prins (1985) showed that  $\text{PO}_4^{3-}\text{-P}$  at concentrations from 0.05 – 1.5 mg/L in wetlands in temperate climate were able to cause phytoplankton bloom, sufficient to lower production of *S. pectinata*.

Te Waihora (Lake Ellesmere) is now a hypertrophic lake, with nutrient input from tributary streams and groundwater flowing through an intensive land use catchment (Hughey and Taylor 2009). In Te Waihora, inorganic N existed mostly in the form of nitrate, with an average of 0.64 mg/L and a maximum of 3.75 mg/L. Inorganic N was in high concentration near the tributary inflows (Selwyn and LII rivers) and the concentration of inorganic N decreased at sites away from these inflows (Lineham 1983). Organic N was largely within phytoplanktons, and was 0.95 mg/L on average and 4.15 mg/L at maximum. Primarily inorganic N was converted into organic N following its input, and organic N decline was observed after lake openings (Lineham 1983). In the 1978-1980 survey, soluble P was 0.010 mg/L on average and ranged from <0.001 to 0.086 mg/L across the lake, whereas total P

was 0.151 on average and ranged from 0.013 to 0.960 mg/ L. Total P was affected by wind-driven sediment re-suspension and was greater in the centre of the lake (Lineham 1983).

TN and TP in sediment cores collected from the centre of Te Waihora demonstrate an exponential decay with depth until a more or less constant level: TP in the surface sediment is 2283 mg/ kg dry wt and decreases to 867 mg/ kg dry wt at 25cm depth, whereas TN decreases from 2841 mg/kg dry wt to 1388 mg/kg dry wt (Trolle 2009). The water retention time in the lake is estimated to be an order of several months and the turnover time for N and P between mineral form and organic form is short (30 to 40 time a year for N) (Lineham 1983). It seems unlikely that *S. pectinata* would be N or P limited in the lake.

Bicarbonate is the primary photosynthetic carbon source for *S. pectinata*, although it also has a high affinity for CO<sub>2</sub> (Sand-Jensen, 1983). Plants die when stems and leaves are cultured without dissolved inorganic carbon (DIC) while roots and rhizomes are supplied with enough DIC (van Wijk, 1989c). The plant needs a minimum of 30.5 mg/L of HCO<sub>3</sub><sup>-</sup> in the water to survive (Huebert & Gorham, 1983). *S. pectinata* has an active bicarbonate uptake mechanism with the lower surface of leaves involved in HCO<sub>3</sub><sup>-</sup> uptake while the upper in OH<sup>-</sup> release (Prins, Snel, Helder, & Zanstra, 1980). van Wijk (1989c) also reported a positive relationship between HCO<sub>3</sub><sup>-</sup> concentrations (from 61 to 311 mg/L) and growth rates of *S. pectinata*. The bicarbonate concentration in the water column of Te Waihora (Lake Ellesmere) was found to vary from 61 to 70 mg/L (Lineham, 1983), easily supporting the growth of *S. pectinata*.

## Substrate

*S. pectinata* has been found on various substrates. The effect of substrate on colonisation of *S. pectinata* is confounded by other factors, such as wave action, water depth and nutrient availability (Madsen, 1986). Types of substrates are consequences of wave action, which in turn is related to water depth and wave size, as fine particles tend to accumulate in more sheltered areas (Sculthorpe, 1967). Culture experiments found that plant growth increased with increasing peat (refractory organic matter) content in the sediment but not with increasing labile organic (glucose or sucrose) content. A possible reason is that the sediment density is lower in peat-amended substrates (Spencer, 1990) and therefore better for root

and rhizome development. Kantrud (1990) suggested that fine clay is less conducive to rooting compared to sand and peat. A positive relationship has been found between the frequency of *S. pectinata* and coarseness of bottom substrates (Schmid, 1965). The coverage of *S. pectinata* is directly related to the amount of sand in the substrate (Kantrud, 1990). Pure sand does not support *S. pectinata* as abundant as silt and gravel (J. D. Madsen, 1986), possibly due to a limited nutrient in the sand. It is possible that provided enough nutrients in sediment, the coarser (within an appropriate range) the substrate is, the more suitable it is for the growth of *S. pectinata*. A substrate consisting sand and clay with a mass ratio of 3:1 was always used for laboratory growth of *S. pectinata* (Hootsmans & Vermaat, 1994b; Pilon & Santamaria, 2002a; Vermaat & Hootsmans, 1994a, 1994b).

### Periphyton Coverage

Periphyton coverage can reduce PAR and has been blamed for the reduction of *S. pectinata* (Schiemer and Prosser 1976, Filbin and Barko 1985). The periphyton coating may consist of epiphytes, desmids and diatoms, bacteria and clay particles (Kantrud 1990). Leaves of *S. pectinata* can be colonized by epiphytes as early as 1.5 weeks after tuber germination and cuticular damage can occur at 6 weeks (Howard-Williams et al. 1978). *S. pectinata* in Wisconsin was found with higher degrees algal coating than other genera of aquatic macrophytes (Gough and Woelkerling 1976) and epiphytes growing on *S. pectinata* can weigh more than its biomass. The most obvious effect of periphyton coverage on growth of the macrophyte is reducing incident light. Laboratory experiments mimicking eutrophic conditions showed that the periphyton coating reached a density of  $0.5 \text{ mg afdw cm}^{-2}$  after 3 to 4 weeks, attenuating 50% of incident light (Vermaat and Hootsmans 1994c). High attenuation of incident light by periphyton coating on *S. pectinata* was found in Lake Veluwe (Vermaat 1994).

### **Key factors in the habitat template**

In Lake Veluwe, the dynamics of *S. pectinata* was regulated by light (water transparency) and the negative effect of periphyton on *S. pectinata* was through light reductions. In Te Waihora

(Lake Ellesmere), high turbidity causes a steep light gradient and reduces photosynthetically available radiation (PAR) in the lower water column, constraining macrophyte growth (Gerbeaux and Ward 1986). Therefore the primary factor in the habitat template is light, which is determined by light attenuation and depth of the water column. Salinity fluctuation is related to the decline and recovery of macrophyte and compounds the growth of macrophyte beds in Te Waihora (Lake Ellesmere) (Gerbeaux 1993). The second key factor in the habitat template is salinity. Temperature fundamentally regulates the growth of plants. However, temperature in Te Waihora seems not to limit the growth of *S. pectinata*, nor does nutrient in Te Waihora based on literature study in the previous section. Thus the effects of temperature and nutrient on the growth of *S. pectinata* are not prioritised to be studied, but are considered when constructing a habitat template.

The habitat template aims to define an appropriate range for incident irradiance, water depth, light attenuation coefficient, and salinity in Te Waihora (Lake Ellesmere), to allow *S. pectinata*, with appropriate acclimations, to grow and persist indefinitely.

## **Outline of the thesis**

In order to construct a habitat template, the research programme determines the extent to which morphological and physiological acclimation (e.g. etiolation and shade adaption) can allow the plants to intercept sufficient light to survive and grow under a range of irradiance levels interacting with fluctuating salinities.

Chapters 2-4 contain most of the research output from the PhD programme, and are written with a view to submit to international journals. Their structure is therefore designed to be self-sufficient, though where necessary cross-references among chapters are included.

Light availability is identified as the most challenging requirement for the survival of *S. pectinata*, thus it is of crucial importance to understand the acclimation of *S. pectinata* in turbid waters. To this end, a series of experiments were undertaken to determine its ability to acclimate to shade conditions. Morphological acclimation was studied by observing the plant growth in a greenhouse under 0 and 70% shading conditions. Physiological acclimation was investigated by comparing photosynthesis vs irradiance (PI) relationships in plants

grown under high and low irradiance levels in three scenarios: greenhouse, growth chamber, and in Te Waihora (Lake Ellesmere). The results of these experiments are described in *Chapter 2*.

In addition to light, salinity and its fluctuation influence the expansion and recession of *S. pectinata* in Te Waihora (Lake Ellesmere). Salinity at a shelter site in Te Waihora (Lake Ellesmere) where *S. pectinata* is often found ranges from 2 ppt to 27 ppt ( $8.4 \pm 3.8$  ppt, mean  $\pm$  standard deviation). To understand the effect of salinity on *S. pectinata*, growth responses of *S. pectinata* to a range of salinities (6, 12, and 20 ppt) were investigated in the laboratory. The resilience of *S. pectinata* to extremely high salinity was studied by following plant growth in freshwater after exposure to an apparently lethal salinity level. Regarding the effects of salinity fluctuations, plant performance through both abrupt and stepwise salt acclimations to 12 ppt was studied. Leaf demography, leaf length, leaf photosynthetic pigments, and leaf photosynthesis-irradiance response were measured. These results are described in *Chapter 3*.

In a turbid water column, leaves at different depths along a surface-reaching shoot would experience a gradient of light intensities from full sunlight at the water surface attenuating to  $50 \mu\text{mol m}^{-2} \text{s}^{-1}$  at 0.2 m below the water surface in Te Waihora (Lake Ellesmere). Given this, how leaves of *S. pectinata* photosynthesize at different depths in the turbid water column, interacting with fluctuating salinities would provide a basic understanding on how shoots of different sizes cope with different water depths and salinity, from an energy harvesting perspective. Laboratory experiments were therefore undertaken to determine leaf photosynthesis at different irradiance levels (corresponding to different optic depth) at various salinity levels. These are presented in *Chapter 4*.

Finally, the findings in the previous chapters are summarised in *Chapter 5*, leading to a habitat template of *S. pectinata* in Te Waihora (Lake Ellesmere). The habitat template is defined in terms of the range of water depth, light attenuation coefficients and salinity regime to allow *S. pectinata* to grow from a sprouting tuber to a surface-reaching shoot (a single growth stage). The habitat template is verified against a field population that was monitored over two growing seasons. The limitations of the habitat template and future

research to allow *S. pectinata* to persist through consecutive growing seasons (indefinitely) are discussed.

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## **Chapter 2: Morphological and physiological acclimations in *Stuckenia pectinata* to low light conditions**

### **Abstract**

The submerged macrophyte *Stuckenia pectinata* was once abundant in the coastal lagoon Te Waihora (Lake Ellesmere) on the east coast of the South Island of New Zealand. For half a century the plant has been rare in the lagoon, which is currently highly turbid. Attempts to reintroduce the plant to the lake require an understanding of how it can perform in the highly turbid water, and to this end, a series of experiments was undertaken to determine its ability to acclimate to shade conditions. Morphological acclimation was studied by observing plant growth in a greenhouse under 0 and 70% shading conditions. Physiological acclimation was investigated by comparing photosynthesis vs irradiance (PI) relationships in plants grown under a range of irradiances in three scenarios: greenhouse, growth chamber, and in Te Waihora (Lake Ellesmere). Morphologically, at low irradiance primary shoots grew taller, produced longer and lighter leaves and senesced leaves more quickly than at high irradiance. Physiologically, PI curves of low light plants demonstrated a strong increase in apparent quantum yield and a slight increase in light-saturated photosynthesis compared to high light plants. Low-light laboratory plants had higher leaf chlorophyll-a concentrations whereas low-light field plants had lower chlorophyll a/b ratios. Both laboratory and field plants had lower chlorophyll-a/ total carotenoid ratios. *S. pectinata*, when low-light stressed in the turbid water column, would elongate stems to reach the upper water column sooner, elongate leaves to increase light interception area, and to adjust photosynthetic pigments to enhance photosynthesis under low light.

**Key Words:** *Stuckenia pectinata*, shade acclimation, stem elongation, leaf senescence, chlorophylls, photosynthesis

## Introduction

Te Waihora (Lake Ellesmere) is a large, shallow and brackish lagoon located on the east coast of the South Island of New Zealand. It is persistently highly turbid, primarily due to high concentrations of fine suspended sediment (Hawes and Ward 1996, Harward and Ward 2009), common in wind-exposed shallow lakes with unconsolidated sediments (Gerbeaux and Ward 1991). Intensification of land use in its catchment area has made the lake hypertrophic and high concentrations of phytoplankton also contribute to the water turbidity (Gerbeaux and Ward 1991, Hawes and Ward 1996).

An extensive submerged macrophyte bed (mainly *Stunkenia pectinata* and *Ruppia spp.*) used to colonize much of the littoral margin of the lake, which reduced shoreline wave action and sediment re-suspension and created a marginal zone of lower turbidity (Harward and Ward 2009). However, an extreme weather event in 1968, known as the “Wahine” storm, completely removed the macrophyte bed. Since then submerged macrophytes have been only occasionally found in shallow and sheltered areas (Gerbeaux 1993, Jellyman et al. 2009).

Insufficient light is considered the main constraint for macrophyte regeneration in the Lake (Gerbeaux and Ward 1991, Jellyman et al. 2009). Light is essential for photosynthesis and therefore for the growth of macrophytes. The amount of light, particularly photosynthetically active radiation (PAR) that penetrates through the water column, determines the maximum depth that macrophytes can colonize (Spence 1982, Hawes et al. 2003). In Te Waihora (Lake Ellesmere), this is complicated by the need for sufficient light to coincide with a zone of the lake that is viable in terms of wave action and water level. Exposure to large wave forces can prevent submerged macrophyte establishments in the shallow margins of large lakes (Hawes et al. 2003, Van Zuidam and Peeters 2015) and is highly correlated to the minimum colonisation depth for macrophyte (Chambers 1987, Stevens and Lacy 2012). Jellyman et al. (2009), based a wave length of 400 cm and empirical equation from Chamber (1987), estimated a minimum colonisation depth of 2.1 m in Te Waihora (Lake Ellesmere) while the euphotic zone was only 0.45 m deep, theoretically leaving no niche for submerged macrophytes. In addition, the level of Te Waihora (Lake Ellesmere) fluctuates from below 0.6 m up to more than 1.2 m over the course of a year, making the task of macrophyte re-colonization Sisyphean.

In order to prevent shore-line erosion, to improve water clarity and to increase habitat biodiversity (Jellyman et al. 2009), there is an aspiration to re-establish a macrophyte bed in Te Waihora (Lake Ellesmere). The intent is that tall, well-established macrophytes will be transplanted into the lake behind a wave barrier, which mitigates the hydrodynamic forces disturbing their establishment, in relatively deep water. High turbidity attenuates light strongly with depth, therefore leaving low light availability in the lower water column for the macrophytes. An understanding of the ability of these species to persist and spread under potentially light-limiting conditions is sought to predict its performance in the turbid lake.

The submerged macrophyte *Stuckenia pectinata* (previously named *Potamogeton pectinatus*) was suggested to be better in tolerance for low water clarity than *Ruppia spp.* in the lake (Gerbeaux and Ward 1991), therefore is the investigated species in this study. *S. pectinata* is a submerged angiosperm, rooted in sediment, and has long stems and small, mostly undivided linear leaves. In favourable conditions, it reproduces vegetatively with tubers, which send up shoots and develop rhizomes that send up secondary shoots in great abundance (Kantrud 1990). These attributes should make the plant well suited to the proposed re-colonization strategy.

Previous studies showed *S. pectinata* in low-light conditions elongated stem length via internode lengths, had less secondary shoots and fewer leaves (Hootsmans and Vermaat 1994, Hootsmans et al. 1996, Pilon and Santamaria 2002a). Physiologically, photosynthesis - irradiance (PI) curves of *S. pectinata* demonstrated contradictory results: enhanced maximum gross photosynthetic rates, increased initial slope of the PI curves and dark respiration (Hootsmans et al. 1996, Pilon and Santamaria 2002a) or decreased maximum gross photosynthetic rates and no changes in initial slope and dark respirations (Hootsmans and Vermaat 1994). Photo-acclimation to low-light increases the amount of light harvesting chlorophyll proteins of photosystem II and photosystem I for maximal light capture, that is inversely related to Chl-a: b ratio (Anderson et al. 1995). Through these acclimations, similar photosynthesis and biomass accrual were found in plants under  $50 \mu\text{mol m}^{-2} \text{s}^{-1}$  PAR compared to ones at  $350 \mu\text{mol m}^{-2} \text{s}^{-1}$  PAR (Hootsmans et al. 1996, Pilon and Santamaria 2002a).

The primary goal of this study is to investigate morphological and physiological acclimations of *S. pectinata* to low-light conditions to determine whether *S. pectinata* is capable of colonising highly turbid waters. Morphological acclimations are associated with total plant light harvesting area and physiological acclimations are associated with light utilisation efficiency and leaf photosynthetic rate (Niinemets 2010, Fu et al. 2012). *S. pectinata* under low-light conditions is hypothesized to have longer stems and fewer leaves, to enhance chlorophyll concentrations and light utilisation efficiency, and to yield similar biomass compared to high-light plants.

## **Material and Methods**

### ***Morphological Acclimation***

#### **Plant material and Growth Conditions**

Tubers of *S. pectinata* were sourced from Halswell River (43.75° S, 172.60° E, New Zealand), a tributary to Te Waihora, in early March 2015 and stored at 4 °C in darkness. In August, the tubers of fresh weight  $34 \pm 2$  mg (mean  $\pm$  standard error) were planted. Every tuber was buried 1 cm deep in a 200 mL, 8 cm tall plastic beaker half filled with sieved fine sediment from Te Waihora (Lake Ellesmere) at the bottom and half with fine sand on top. Pots were submerged in 10 cm of tap water ( $\text{PO}_4^{3-} = 0.1 - 0.13$  mg /L;  $\text{NO}_3^- - \text{N} = 1.0 - 1.1$  mg /L) and incubated in natural light conditions in a greenhouse at Lincoln University, NZ. Almost all tubers germinated within 3 weeks, and 32 germinating tubers were transferred to a fibreglass tank (2.6 m in diameter and 1.2 m in height) with 0.6 m deep water. Half of the water was replaced weekly with fresh tap water. Air was bubbled continuously into the water by four air pumps (each 4 L/min, ACO-2005, HAILEA®). Three submerged pumps (23 w, 1001, EHEIM, GmbH) gently moved water clockwise. Through the experiment, water temperature was between 15 and 20°C, turbidity was under 2 NTU (Nephelometric Turbidity Units), and morning pH was between 6.7 and 7.7 (PHE-7352-15 pH Sensor, Omega, US).

One green polyethylene 70% shade cloth (100 cm x 60 cm) was positioned 10 cm above the water surface for shading, the shaded area was a low light (LL) treatment, compared to non-shaded space in the same aquarium as high light (HL) treatment, where sprouting tubers would use light. To prevent light scattering in the tank that diffuses the shading effect at the

bottom of the tank, white plastic baskets were placed under the shade as well as in the HL treatment, to ensure that the plants placed in the baskets primarily received light from above. On sunny days, underwater PAR (at 25 cm deep) peaked on average around 520  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  (12:00 -14:00) for the HL treatment and around 150  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  for the LL treatment (Li-193 Spherical Quantum Sensor and Li-1700 Data-logger Li-Cor, Inc., US). Day length through the experiment averaged 11 hours. Initially, 16 small plants were assigned to each treatment. In the end, 12 plants in the LL treatment and 10 in the HL treatment remained intact and those plants were used for morphological observations.

### Plant Morphology and Biomass

Plants were observed every 6 days after treatments started and at each time the stem length, number of internodes, length of every green leaf, sum of green leaf lengths, number of green leaves as well as the number of senesced leaves of the primary stem were recorded. A green leaf was registered when more than half of its leaf total length was green and healthy, otherwise, it was recorded as a senesced leaf. A newly produced leaf was not recorded until it was fully expanded. The number of days between the first appearance and the senescence of a leaf was termed the leaf longevity. Plants were measured directly on day 1, 6, 12, 18 and 24 and were measured by photographing and image processing after day 24. When photographing, *S. pectinata* was flattened on a gridded board that was 5 mm below the water surface, and photos were taken with a camera (Leica X2) from 50 cm above the water surface. The photos were orthogonally transformed (Adobe Lightroom 5) then analysed (Image J). *S. pectinata* was not easily flattened when small and was thus measured with a ruler.

Along with measurement of the main shoot morphology, the number of secondary shoots was counted and the largest secondary shoot of each plant was photographed on day 60, and analysed for the morphological characteristics.

On day 61, 9 LL plants and 6 HL plants were harvested, washed free of sediment in tap water, and dissected into leaves, stems and belowground parts. Every leaf and stem was measured for fresh weight (FW) and all parts were measured for dry weight (DW). For each plant, total DW and belowground: total DW ratio was calculated, and the number of tubers

was counted. DW: FW ratios were calculated for stems and leaves. Fresh weight was determined after blotting plant parts with tissue paper and DW determined after drying at 80 °C for 24 hours.

### ***Leaf Photosynthetic Acclimations***

#### **Plant material**

Photosynthetic acclimation of *S. pectinata* to irradiance was investigated under three scenarios, greenhouse, field populations and growth chamber.

The greenhouse scenario used *S. pectinata* plants cultured for the morphological observations described above as LL treatment and HL treatment. Before the plant harvest at the end of the experiment, one green healthy fully expanded leaf per plant on the main shoot from randomly chosen 9 LL plants and 6 HL plants were collected for analysis of photosynthesis vs irradiance (PI) relationships.

The second scenario was in Te Waihora (Lake Ellesmere) at Timber Yard Point, where a small population of *S. pectinata* still occurs. Incident PAR and light attenuation coefficient at the site was surveyed monthly. Incident PAR was measured using a LiCor Quantum Sensor and recorded using a Li-1500 Data-logger (Li-Cor, Inc., US) during the solar noon period. The solar-noon time for the site was taken from a website (<http://dateandtime.info>). The attenuation of down-welling PAR with water depth was estimated from underwater PAR at 0, 8, 16, and 24 cm, recorded using a LiCor underwater Quantum Sensor. Light attenuation coefficient ( $K_d$ ) was estimated by log-linear regression of depth and PAR, according to Kirk (2011). In March 2016, plants, standing in 25 °C and 45 cm deep water, were collected and stored in ambient lake water, transferred back to the laboratory and kept in the dark. On the sampling day, the salinity was 6 ppt. Surface-reaching leaves were sampled as high light acclimated, whereas leaves submerged at 25 cm deep were taken as low light acclimated. The next day, one green healthy leaf per shoot, from each of 6 surface reaching shoots and 6 submerged shoots, were analysed for PI relationships.

The third scenario used a controlled growth chamber, where *S. pectinata* were acclimated to two PAR levels, 50 and 200  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Irradiance was supplied by LED lights, and the



chamber was maintained at 20 °C for the duration of the 3-week experiment. Plants were originally sourced from a wild population in a tributary river that drains to Te Waihora (Lake Ellesmere) in February 2015 and kept in the greenhouse for a month before moving into the growth chamber. In the growth chamber, five replicate plants for each light treatment were cultured in separate plastic containers with 4 L water (0.25 m deep). After three weeks of acclimation, a healthy leaf at water surface was collected from each plant for PI analysis.

### Photosynthesis-Irradiance (PI) analysis

For each leaf collected, oxygen production rates were measured in the dark and under a series of increasing PARs (Table 1). Darkness was created by wrapping samples with aluminium foil. Irradiance was provided by three, radially arranged 100 W LED floodlights (colour temperature 4000 K) (Photo 1), placed 50 cm above a temperature-controlled water bath. The different irradiance levels were created using different layers of white translucent cloth and two polycarbonate boards placed 30 cm below the LED lights. Two polycarbonate boards were placed in-between white cloth to increase light reflection and scattering as the light pass through, therefore the light field was more homogeneous (Table 1) for plant incubations in the water bath. A LiCor Li-192 quantum sensor was used to measure PAR. The gradient of PARs achieved was 21, 46, 66, 89, 129, 192, 242, and 317  $\mu\text{mol m}^{-2} \text{s}^{-1}$ .

Each element in the water bath (the water bath container, the water pump, the heating unit, and the rack) was black to prevent reflections. A circular area, 23 cm in diameter at the surface of the water bath, was illuminated almost homogenously (Table 1) and could accommodate up to 22 glass vials for incubation on a rack 1 cm below the water surface (Photo 2). The water bath was maintained between 20.4 and 20.8 °C and circulated using a submersible pump (23 w, 1001, EHEIM, GmbH).



Photo 1: The apparatus for photosynthesis measurement (an illumination platform and a sample handling water bath)

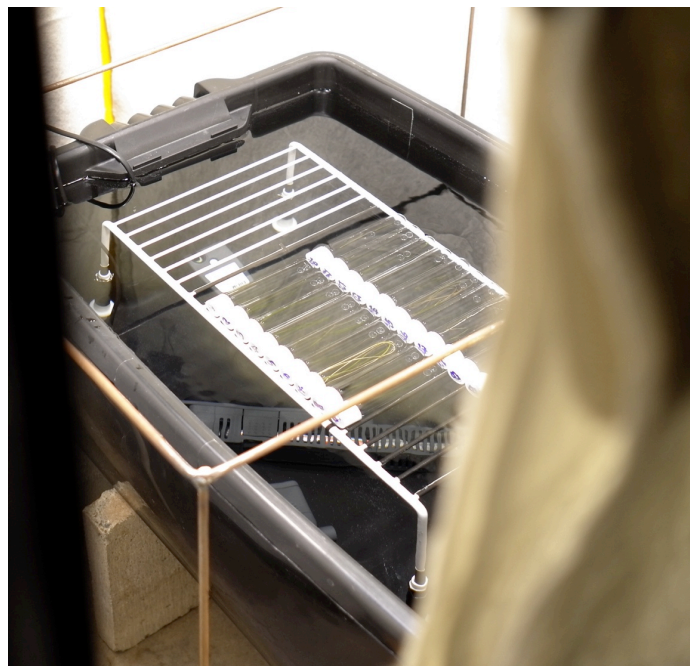


Photo 2. The leaf samples in incubation in the water bath under illumination (inside the illumination platform)

Incubations were of a single leaf sealed in a 12.2 mL glass vial, filled with air-saturated tap water, enriched with 10 mmol/L bicarbonate to prevent carbon depletion affecting the photosynthetic rate. Incubations of leaf samples collected from the lake used tap water adjusted to 6 ppt with artificial sea salt. Three blank glass vials as controls were incubated

each time along with leaf samples. At the end of each incubation, the glass vials were well mixed (two 5 mm diameter glass beads had been placed in each vial to assist in-vial water mixing), and oxygen concentrations were estimated using a PreSens Oxygen Microsensor connected to a Microx 4 control unit (PreSens GmbH, Germany). Oxygen production was determined as the difference from the controls at the end of each incubation. Initial incubations were in darkness, after which the leaves were incubated incrementally over a gradient of irradiance (Table 1). Because the incubations of leaf samples started with air-saturated water for easy handling, incubation time was shortened with increasing actinic light levels (Table 1) to prevent air saturation above 140%. When saturation in incubation vials exceeds 155% during the methodology development, air bubble started to form and under-estimate oxygen concentration. The leaves collected from the greenhouse and from the lake were incubated from light level 1 to 8, while the growth chamber leaves were only incubated at light level 1 to 6.

Table.1 PAR treatments and incubation times used for determination of photosynthesis-irradiance relationships. (For each PAR treatment, incubation area PAR was presented with mode and range)

Light Level	1	2	3	4	5	6	7	8
Mode ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )	21	46	66	89	129	192	242	317
Range ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )	2.6	3.6	1.8	1.8	3.0	3.0	9.0	14.0
Incubation time (s)	3600	3600	2700	2700	1800	1800	1800	1800

After all incubations were complete, leaves were dried at 80 °C for 24 hours, cooled and their DW were determined. For each leaf, oxygen production rate at each light level was calculated as the rate of change of oxygen concentration, normalised to leaf dry weight, yielding units of  $\mu\text{g O}_2 \text{g}^{-1} \text{DW s}^{-1}$ . Rates were plotted against irradiance for curve fitting.

Although Hootsmans and Vermaat (1994) concluded there is no objective criterion to prioritize one model over others in describing P-I curves, empirically and subjectively the Hyperbolic Tangent Model (Jassby and Platt, 1976), modified to include a respiration parameter, was chosen in this study.

Photosynthesis - Irradiance Model:

$$P = P_{\max} * \tanh (\alpha * I / P_{\max}) + R$$

Where:

**P**: net photosynthetic rate, as a dependent variable

**I**: photosynthetically active radiation, as an independent variable

**$\alpha$** : apparent quantum yield, the slope of the curve at low irradiance before the onset of maximum photosynthesis

**$P_{\max}$** : light-saturated (maximum gross) photosynthetic rate

**R**: dark respiration rate

Hyperbolic Tangent Models were fitted with the Analytic Gauss-Newton regression algorithm (JMP 9, SAS®), and the three key parameters ( **$\alpha$** ,  **$P_{\max}$** , and **R**) were estimated simultaneously.

## Photosynthetic Pigments

Photosynthetic pigments in leaves of *S. pectinata* in low vs. high light conditions were investigated in plants collected from Te Waihora (Lake Ellesmere) and in plants grown in the growth chamber as described above. For the lake-grown plants, four surface-reaching (high light) and four submerged (low light) plants were collected, stored in ambient lake water, and transferred to the lab. Immediately a healthy looking, fully expanded leaf from each plant was excised for pigment analysis. For the growth chamber grown plants, after acclimation to PARs of 50 and 200  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , one healthy leaf from each plant, with 5 replicates per group, was collected for pigment analysis.

Pigments were analysed within an hour after leaf collection. Each leaf was ground with a mortar and a pestle in 5 mL 90% acetone for 30 seconds, before the extracts were transferred into a 15 mL plastic vial. This step was repeated twice to ensure a complete extraction. After storing at 6°C for 24 hours, the extracts were centrifuged at 3000 r.p.m for 15 minutes. The absorbance of the supernatant was then measured at 470 nm, 647nm, 664nm, and 750nm (Spectrophotometer DR3900, HACH®, USA) in a 1 cm glass cuvette. Chlorophyll-a and chlorophyll-b concentrations were calculated according to Porra (2002)

and chlorophyll-a:b ratio was calculated. Total carotenoid concentration was estimated according to Wellburn (1994).

### ***Data Analysis***

In this study, time series data (repeated measurements) were analysed using a univariate linear model with plants nested in treatment (repeated measures ANOVA) followed by least square mean Tukey HSD test. Residual normality of the fitted linear model was checked using Shapiro-Wilk test (Shapiro and Wilk 1965). If residuals were not normally distributed, data were transformed for repeated measures ANOVA, otherwise multivariate linear model (multivariate analysis of variance, MANOVA) was employed.

Main stem length over time was analysed using MANOVA. At each sampling time, within treatment normality was checked using Shapiro-Wilk test and equal variance was checked between treatments using Levene test (Levene 1960). Main stem lengths were all normally distributed and were of equal variance except on day 18 and day 24, where samplings were excluded from the MANOVA test. Due to the data pattern, time series data of total lengths of green leaves, number of green leaves, and the number of cumulative senesced leaves at 6 days intervals before and after day 30 were analysed separately. Data before day 30 were analysed using MANOVA, whereas data after day 30 were analysed using repeated measures ANOVA and post Tukey HSD test. Before MANOVA analysis, within treatment normality and equal variance between treatments on each day were confirmed. In addition, least square mean number of dead leaf on day 48, 54, and 60 were compared using student t test.

Lengths of leaves of different order were considered as independent variables; therefore leaf lengths between light-treatments were compared using t-test assuming non-equal variance after normality was confirmed (Shapiro-Wilk test). Leaves of orders 1 to 5 (Figure 1) were compared separately at each leaf order level and leaves of order 6 to 10 were grouped together for comparison.

Leaf longevity between treatments was compared with a Chi-square test. After normality was confirmed, numbers of secondary shoots, morphological characteristics of the largest secondary shoots on day 60, plant dry matter, dry matter to fresh biomass ratio and

belowground to total biomass ratio, estimated PI curve parameters and pigment concentrations, were compared t-test assuming non equal variance, while non-normal data were compared with non-parametric Wilcoxon Test. All test are performed with JMP 9.0 (SAS®).

## Results

### *Morphological Acclimation*

#### Stem length, leaf length, and number of leaves for the main shoots

During the time frame of this experiment, the main shoot elongated as time progressed, and new leaves were produced continuously from the apical meristem. Having reached its maximum length, a newly produced mature leaf can last for a few weeks before turning brown and senescing. A senesced leaf may detach from the main shoot (leaf 1 in figure 1). As main shoots grew, plants developed secondary shoots (Figure 1).

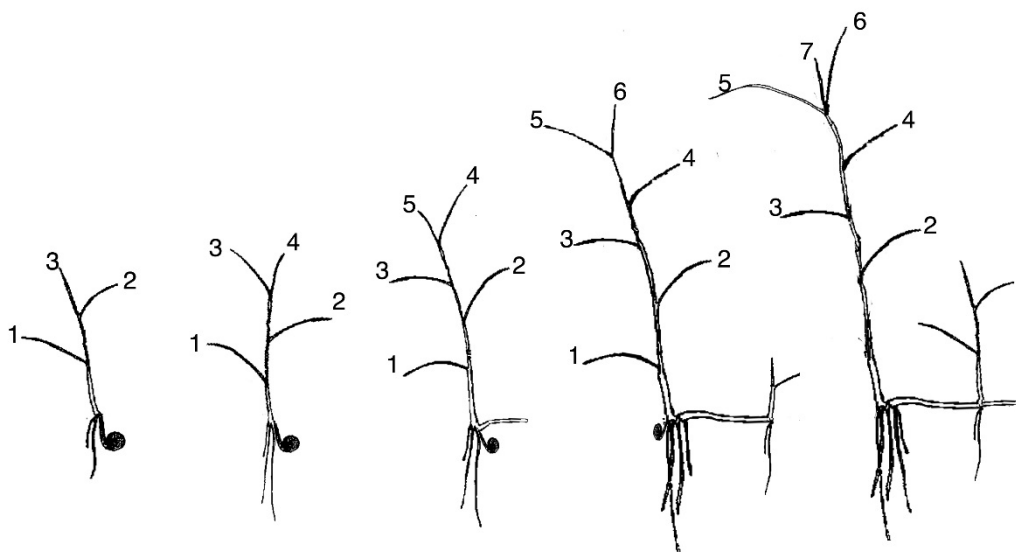


Figure 1. The growth pattern of *Stukenia pectinata* in this study (numbers indicate leaf order for leaves on main shoots)

The stem length of main shoots increased with time (MANOVA,  $p < 0.001$ ), was longer for the LL plants than the HL plants (MANOVA,  $p = 0.013$ ), but was not affected by treatment and time interaction (MANOVA,  $p = 0.064$ ) (figure 2a). On day 60, the number of nodes on

the main stem did not differ and were  $10.1 \pm 0.3$  (mean  $\pm$  standard error) for the LL group and  $10.2 \pm 0.3$  for the HL group.

Before day 30, total green leaf length of the main shoots increased with time (MANOVA,  $p < 0.001$ ), was longer for the LL plants than the HL plants (MANOVA,  $p = 0.036$ ), and was affected by light treatment and time interactions (MANOVA,  $p = 0.049$ ). After day 30, total green leaf length did not differ with time (repeated measure ANOVA,  $p = 0.07$ ) nor differ between treatments (repeated measure ANOVA,  $p = 0.50$ ) (figure 2b). Although treatments and time interactions affected total green leaf length after day 30 (repeated measure ANOVA,  $p < 0.002$ ), no difference between treatments was found on any sampling day.

The 1<sup>st</sup> leaf and 2<sup>nd</sup> leaf were similar in length; the 3<sup>rd</sup> leaf and 4<sup>th</sup> leaf were significantly longer for the LL plants than the HL plants, and leaf 6 to 10 were significantly longer for the LL plants ( $186 \pm 3$  mm) than the HL plants ( $172 \pm 3$  mm) (t-test,  $p = 0.001$ ) (figure 3). The 1<sup>st</sup> and 2<sup>nd</sup> leaf were grown before treatment had started. The LL plants also had a lower dry weight to fresh weight ratio ( $5.1 \pm 0.2\%$ ) for the leaves than the HL plants ( $6.9 \pm 0.1\%$ ) (t-test,  $p < 0.001$ ).

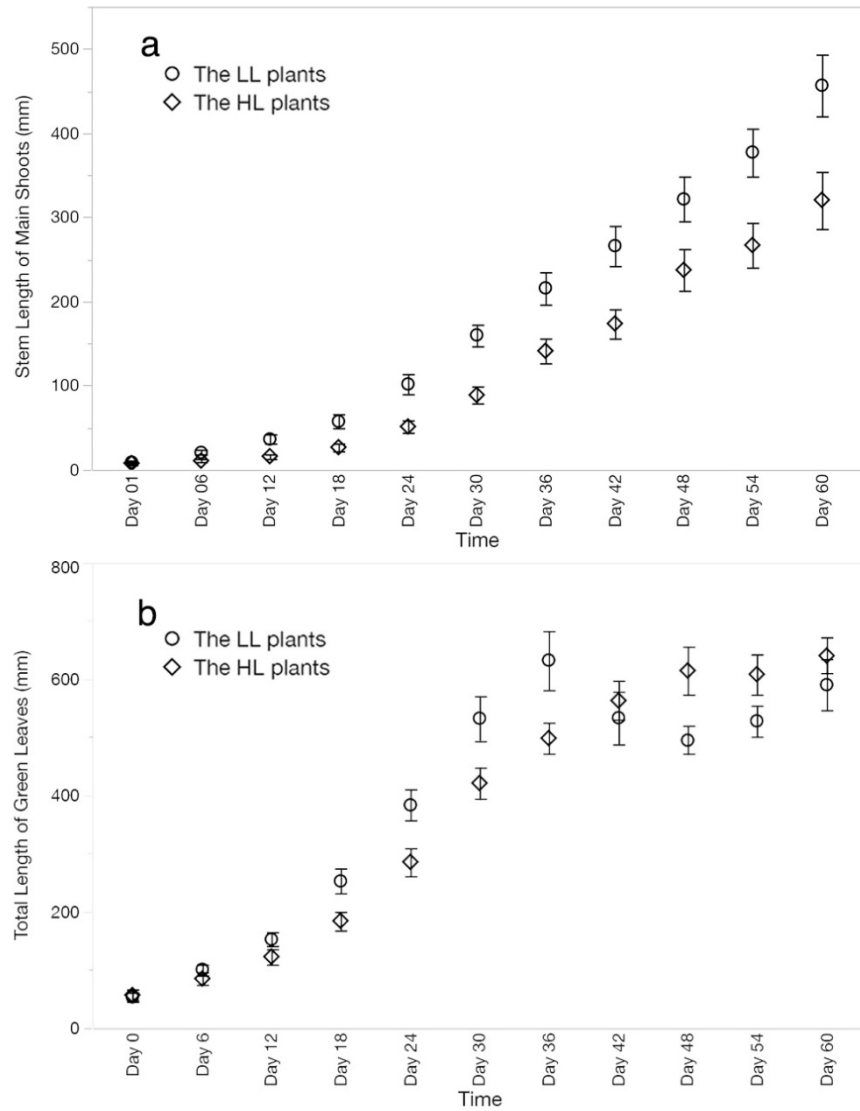


Figure 2. (a) Stem length of the main shoots over time; (b) Total length of green leaves of the main shoots overtime (Data are presented as mean  $\pm$  standard error)

The number of green leaves on the main shoots increased from  $2.1 \pm 0.1$  on day 1 to  $5.1 \pm 0.2$  on day 30 (MANOVA,  $p < 0.01$ ) and the two groups were not different before day 30 (MANOVA,  $p = 0.28$ ), nor were there any time and treatment interactions (MANOVA,  $p = 0.58$ ). During the same period, plants had no or only one senesced leaf, therefore demonstrated no difference between treatments (MANOVA,  $p = 0.26$ ) or among sampling times (MANOVA,  $p = 0.08$ ).



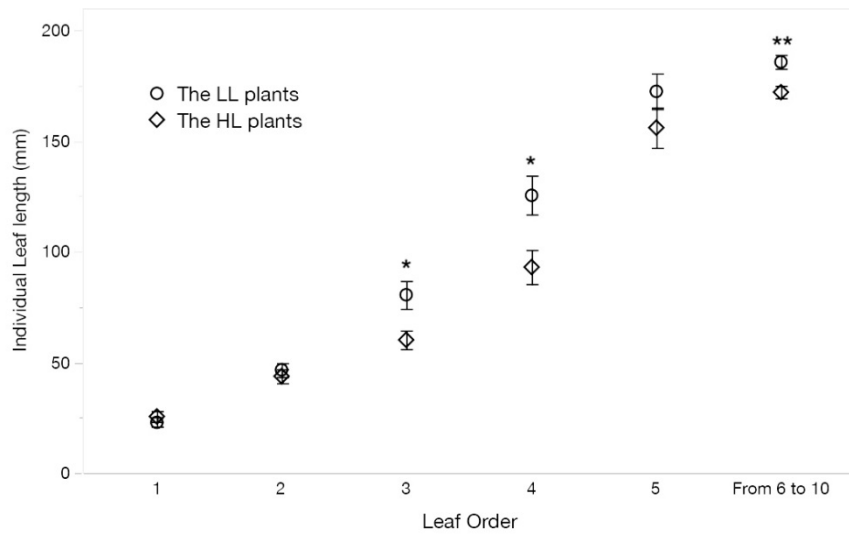


Figure 3. Leaf Length by leaf order (Data are presented as mean  $\pm$  standard error; Significant difference level: “\*”,  $p$ -value  $\leq 0.05$ , “\*\*”,  $p$ -value  $\leq 0.01$ ,  $n = 22$  for each leaf order)

From day 36 to 60, green leaf number was higher for the high light plants ( $4.5 \pm 0.2$ ) than the low light plants ( $3.9 \pm 0.2$ ) (repeated measure ANOVA,  $p = 0.04$ ), and was affected by time (repeated ANOVA,  $p < 0.001$ ). Green leaf number decreased between day 36 and day 42 and did not change with time after day 42 (Figure 4a). Within the same time frame, leaf senescence accelerated and cumulative senesced leaf number increased with time (repeated measure ANOVA,  $p < 0.001$ ), cumulative senesced leaf number continued increasing from day 42 to day 60 (Least Square mean Tukey HSD test,  $p < 0.05$  at each comparison between adjacent sampling times). In the end, LL plants had higher cumulative senesced leaf number on day 48, day 54 and day 60 (Least mean student  $t$  test,  $p < 0.05$ ).

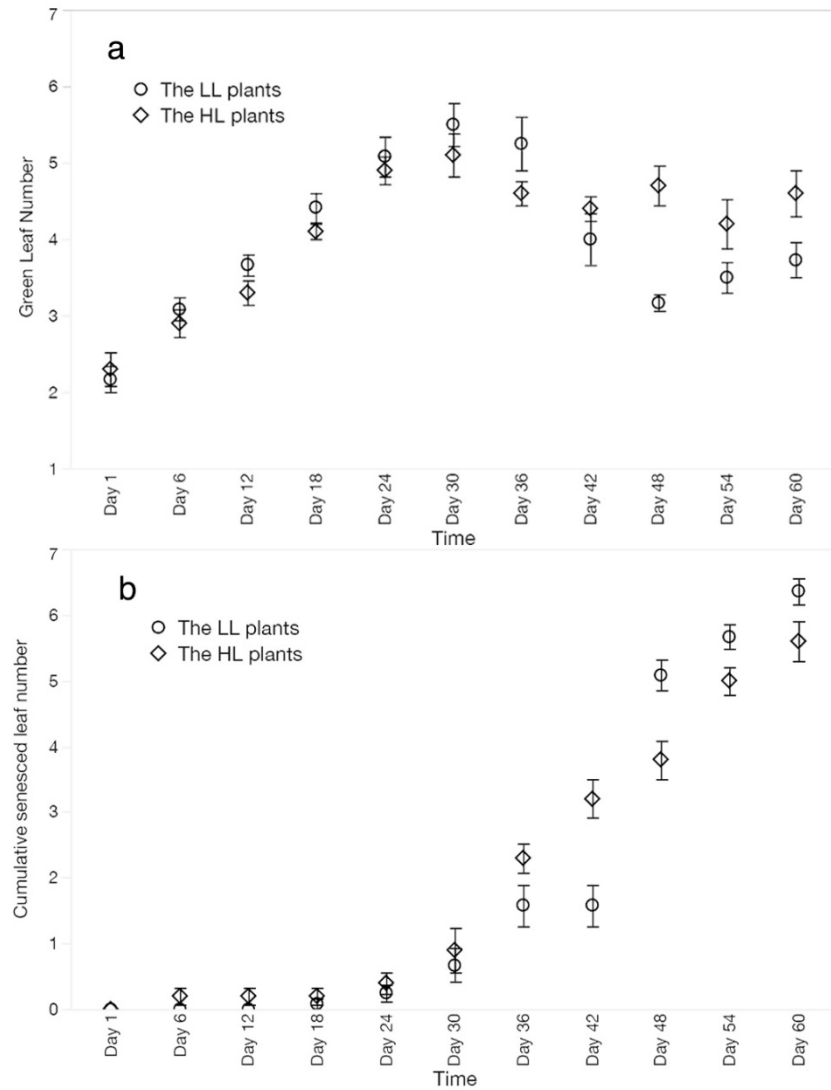


Figure 4. (a) Number of green leaves on the main shoots; (b) Cumulative number of senesced leaves; (Data are presented as mean  $\pm$  standard error)

### Leaf longevity

Leaf longevity could only be recorded for leaves of order 3, 4, and 5 as only these leaves revealed a full life cycle within the time frame of this study. Leaf longevity was 24 or 30 days for the majority of the leaves and there was no significant difference in leaf longevity between the two groups (Chi-square test,  $p = 0.07$ ). However, the LL plants tended to have shorter leaf longevity (figure 5).

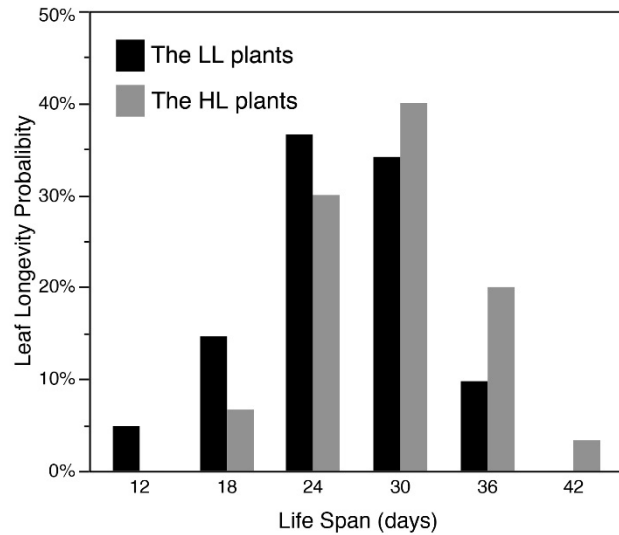


Figure 5. Histogram of leaf longevity of the 3<sup>rd</sup> 4<sup>th</sup> and 5<sup>th</sup> leaves

## Secondary shoots

At the end of the experiment, the LL plants had significantly fewer secondary shoots than the HL plants (Wilcoxon Test,  $p = 0.001$ ). The median number was 1.5 for the LL plants and 3.5 for the HL plants. A comparison of the largest secondary shoots between the two treatments revealed that LL plants had shorter stem length, total green leaf length, averaged leaf length and fewer leaves. In brief, the secondary shoots of the LL plants were much smaller in size compared to the HL plants.

Table 2. Morphological characteristics of the largest secondary shoots on day 60 (Data are presented as mean  $\pm$  standard error except that leaf number is the median of each group; significant difference level: “NS”,  $p$ -value  $> 0.05$ ; “\*”,  $p$ -value  $\leq 0.05$ ; “\*\*\*”,  $p$ -value  $\leq 0.01$ )

	LL plants	HL plants
Stem Length (mm)	156 $\pm$ 37 <sup>*</sup>	290 $\pm$ 49
Total Green Leaf Length (mm)	289 $\pm$ 60 <sup>**</sup>	523 $\pm$ 55
Averaged Leaf Length (mm)	79 $\pm$ 8 <sup>*</sup>	106 $\pm$ 5
Leaf Number	3.5 <sup>**</sup>	6

## Biomass, number of tubers and dry matter to fresh weight ratios

At the end of the growth period, the LL plants not only had less dry matter than the HL plants (t-test,  $p < 0.001$ ), but also had a lower ratio of belowground to total biomass (t-test,  $p = 0.004$ ). The HL plants had a total dry biomass of  $0.15 \pm 0.01$  g and the belowground biomass accounted for  $34 \pm 4$  % of the total biomass, whereas the LL plants had a total dry biomass of  $0.03 \pm 0.003$  g and the belowground part accounted for  $20 \pm 2$  %. The LL plants also had produced fewer tubers than the HL plants, with an average of 0.4 and 2.7 for the LL and HL groups, respectively.

## Leaf Photosynthetic Acclimation

### Photosynthesis – Irradiance curves

In the three scenarios, PI curves of the low light acclimated leaves showed higher  $P_{\max}$  and alpha on average than HL plants, but did not differ in dark respiration (Figure 7). Alpha in all scenarios was significantly higher for the low light acclimated leaves, whereas  $P_{\max}$  was only significantly higher in greenhouse plants (Table 3).

Table 3. The estimated parameters from photosynthesis – irradiance curves of leaves of *S. pectinata* in three scenarios. Data are presented as mean  $\pm$  standard error. Parameters were compared within each scenario (significant difference levels: “NS”,  $p$ -value  $> 0.05$ ; “\*”,  $p$ -value  $\leq 0.05$ ; “\*\*\*”,  $p$ -value  $\leq 0.01$ )

Scenarios		$P_{\max}$ ( $\mu\text{g O}_2 \text{ g}^{-1} \text{ DW s}^{-1}$ )	Alpha ( $\mu\text{g O}_2 \text{ m}^2 \text{ g}^{-1} \text{ DW}$ $\mu\text{mol}^{-1} \text{ photons}$ )	R ( $\mu\text{g O}_2 \text{ g}^{-1} \text{ DW s}^{-1}$ )
Greenhouse	LL plants	$8.77 \pm 0.41^{**}$	$0.060 \pm 0.002^*$	$-0.19 \pm 0.08^{\text{NS}}$
	HL plants	$6.34 \pm 0.56$	$0.051 \pm 0.003$	$-0.29 \pm 0.06$
The Lake	Submerged	$4.95 \pm 0.38^{\text{NS}}$	$0.044 \pm 0.005^*$	$-0.41 \pm 0.03^{\text{NS}}$
	Surface	$4.26 \pm 0.40$	$0.024 \pm 0.002$	$-0.36 \pm 0.03$
Growth Chamber	LED 50	$4.59 \pm 0.50^{\text{NS}}$	$0.053 \pm 0.005^*$	$-0.33 \pm 0.06^{\text{NS}}$
	LED 200	$4.16 \pm 0.37$	$0.026 \pm 0.001$	$-0.46 \pm 0.07$

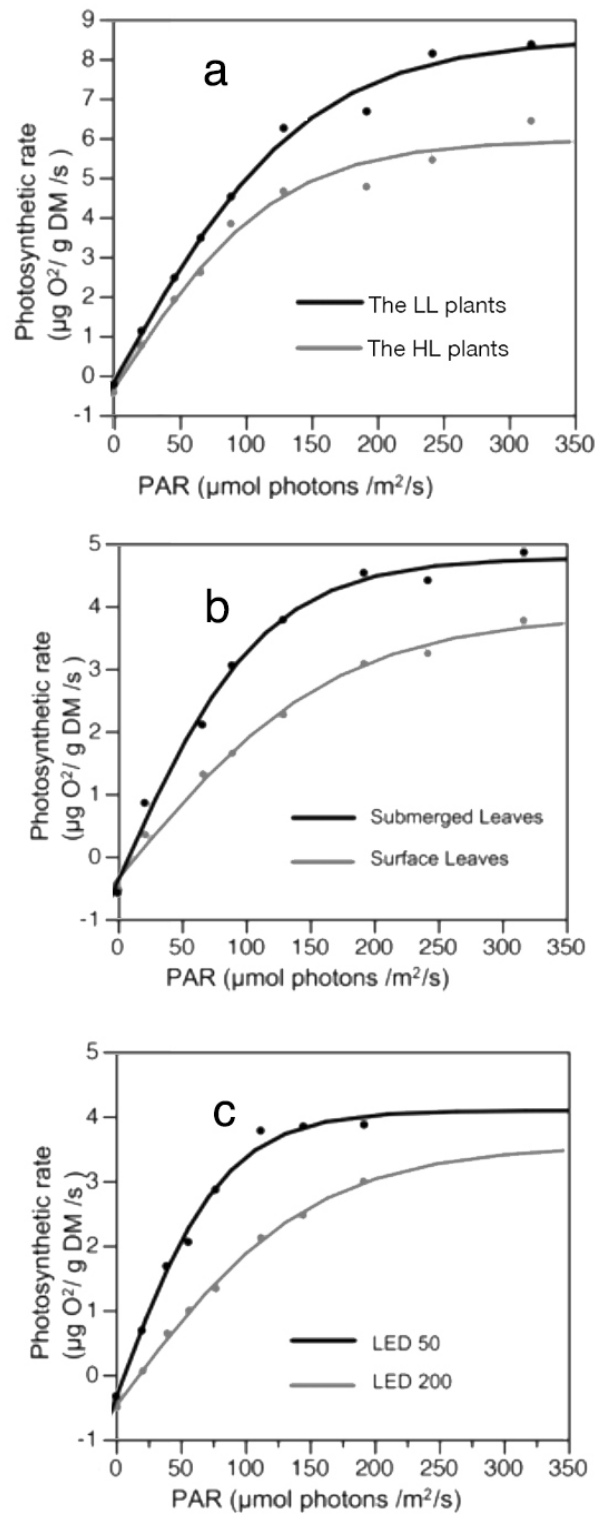


Figure 7. Photosynthesis – Irradiance curves for fully-grown leaves in low vs. high light conditions under three scenarios: the greenhouse (a), the lake (b) and the growth chamber (c) (data points are the average of each group, curves are fitted with the hyperbolic tangent model)

## Pigments

In the lake where plants were sampled, the seasonal mean light attenuation coefficient was  $11.8 \text{ m}^{-1}$ , meaning that the submerged leaves at 25 cm deep received 5% of the PAR the surface leaves received. The mean solar-noon surface PAR was  $1412 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$  at the sampling day. In acclimation to low-light conditions, plants in the lake demonstrated reduced chlorophyll-a:b ratios, whereas growth chamber plants had higher chlorophyll-a concentrations (Table 4). Both of them showed higher chlorophyll-a to carotenoid ratios in comparison to the high-light leaves (Table 4).

Table 4. Concentrations of chlorophyll-a, total carotenoid and chlorophyll-a:b ratios for leaves of *S. pectinata* in lake and in growth chamber. Data are presented as mean  $\pm$  standard error. Parameters were compared only within each scenario and significant difference level are: “NS”,  $p$ -value  $> 0.05$ ; “\*”,  $p$ -value  $\leq 0.05$ ; “\*\*\*”,  $p$ -value  $\leq 0.01$ )

Scenarios		Chlorophyll-a ( $\mu\text{g} / \text{mg DM}$ )	Chlorophyll-a to total carotenoid ratio	Chlorophyll-a:b ratio
Lake	Submerged	$13.1 \pm 1.5^{\text{NS}}$	$3.8 \pm 0.1^{***}$	$2.73 \pm 0.03^{**}$
	Surface	$10.9 \pm 0.5$	$2.0 \pm 0.1$	$2.91 \pm 0.03$
Growth Chamber	LED-50	$18.2 \pm 1.6^{**}$	$3.8 \pm 0.4^{**}$	$2.75 \pm 0.05^{\text{NS}}$
	LED-200	$8.7 \pm 1.3$	$1.7 \pm 0.1$	$2.75 \pm 0.04$

## Discussion

### *Morphological acclimation*

In this study, the main shoots of *Stuckenia pectinata* grown in shade produced longer stems via elongation of internodes, consistent with the previous studies on this species (Vermaat and Hootsmans 1994a, Hootsmans et al. 1996, Pilon and Santamaria 2002a). Shoot elongation has also been shown as acclimation to low light conditions for other plants in the *Potamogeton* genus: *P. maackianus* (Fu et al. 2012, Chen et al. 2016), *P. malaianus* (Fu et al. 2012), and *P. perfoliatus* (Asaeda et al. 2004, Sultana et al. 2010, Toth et al. 2011), also due to longer internode length (Toth et al. 2011). Longer stem length is the first morphological

acclimation in low-light conditions that is particularly beneficial in turbid waters, where longer stem helps plants reach the water surface sooner.

Initially, longer leaf length in the low-light condition resulted in a greater total leaf length of the main shoots, which was offset by faster leaf senescence after day 36. Leaf width was not routinely measured in this study, as pilot experiments had shown this not to vary under light treatments. Therefore, the initial larger total leaf length before day 36 likely represented a larger photosynthetic leaf area. The leaf dry weight to fresh weight ratio was also lower for the shaded plants, suggesting that leaf etiolation involves an energy-conservative way to enhance leaf area. Similarly, *P. perfoliatus* also produces larger and lighter leaves in a shaded environment compared to open waters (Toth et al. 2011). Producing longer leaves with a lower DW: FW ratio is the second morphological acclimation in *S. pectinata* to low-light conditions, which increases the photosynthetic leaf area and helps the plant to reach the water surface more quickly.

Although leaves of the same order were longer, the number of healthy leaves became less for the shaded plants after day 42 (Figure 4a) due to faster leaf senescence rates (Figure 4b) or shorter leaf longevity (Figure 5). The opposite, increased leaf longevity in low-light conditions has been reported for terrestrial plants (tropical pioneer trees, temperate herbs, deciduous trees, and temperate broad-leaved and coniferous evergreens) (Ackerly and Bazzaz 1995, Mojzes et al. 2003). On the contrary, both Woledge (1972) and Ackerly and Bazzaz (1995) reported that severe shading reduced leaf longevity for ryegrass *Lolium perenne* L. and a tropical pioneer tree *Heliocarpus appendiculatus* respectively, whereas less severe shading did not. No finding on leaf longevity of aquatic plants has previously been documented. Assuming the shade-acclimation strategy in both aquatic and terrestrial plants are the same, reduced leaf longevity in *S. pectinata* perhaps is a translation of the overwhelming pressure to use resources to reach the upper water column. In our experiment, plants weren't getting higher irradiance after growing taller under the shade. In natural turbid waters, however, growing taller would result in an exponential gain in irradiance interception, and leaf longevity may not have been reduced if sufficient energy was gained through this strategy.

The morphological plasticity of the main shoots was not shown in the largest secondary shoots, which had shorter stems and leaves, and fewer leaves under low light. Both size and the number of secondary shoots were smaller for the LL plants, suggesting a limitation in the amount of carbon available for secondary shoot development, in line with findings in Vermaat and Hootsmans (1994a). By the end of experiments, *S. pectinata* had accrued less biomass and had a lower belowground to total biomass ratio in low light stress, compatible with the previous studies (Vermaat and Hootsmans 1994a, Pilon and Santamaria 2002a). The reduction in belowground biomass fraction can be attributed to higher investment in leaves and stems at the expense of root and rhizomes (Pilon and Santamaria 2002a). Reduction in resource allocation to roots in response to lower light conditions were also reported for other aquatic plants *P. maackianus*, *P. malaianus*, *V. natans* (Fu et al. 2012). Prioritising stem and leaf extension to increase photosynthetic leaf area at the sacrifice of root development is the key morphological acclimation for *S. pectinata* under low-light stress.

### ***Photosynthetic acclimations***

After acclimation to relatively low-light conditions, leaf photosynthesis of *S. pectinata* demonstrated similar or higher light-saturated photosynthesis, higher apparent quantum yield than those acclimated to high light. However, no change in dark respiration between treatments was found. Previous studies on *S. pectinata* reported photosynthesis-irradiance curves for either whole plants (Hootsmans and Vermaat 1994, Hootsmans et al. 1996) or whole shoots (Pilon and Santamaria 2002a). The plant's growth conditions in the study of Pilon and Santamaria (2002a) was similar to the growth chamber condition in our study (both under  $50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  artificial light with day-length of 12 or 13 hours). Pilon and Santamaria (2002a) reported  $P_{\text{max}}$  for shoots was  $2.5$  to  $3.5 \mu\text{g O}_2 \text{g}^{-1} \text{DW s}^{-1}$  and leaf biomass accounted for 60 % to 80 % of the shoot biomass. Assuming stem as non-photosynthetic tissues, the leaf  $P_{\text{max}}$  is estimated to be  $4.2$  to  $4.4 \mu\text{g O}_2 \text{g}^{-1} \text{DW s}^{-1}$ , comparable to leaf  $P_{\text{max}}$  of growth chamber plants in this study ( $4.6 \mu\text{g O}_2 \text{g}^{-1} \text{DW s}^{-1}$ ). In addition, both our study and Pilon and Santamaria (2002a) showed slightly higher light-saturated photosynthesis and strong increases in apparent quantum yield in low-light



acclimated plants. Enhanced dark respirations in the study of Pilon and Santamaria (2002a) could be attributed to that enhanced stem elongation in low light increased biomass fraction of stem, and therefore increased dark respiration of the whole shoot. The photosynthetic performance of *S. pectinata* in our study is in line with the previous studies.

For both growth chamber and field plants, acclimations to low-light conditions involved increases in leaf chlorophyll concentrations and chlorophyll to carotenoid ratios, and lower chlorophyll-a: b ratios. Higher chlorophyll concentrations in lower light conditions are common for aquatic plants, as reported for *E. densa*, *E. nuttallii*, *M. heterophyllum* (Hussner et al. 2010), *P. perfoliatus* (Asaeda et al. 2004), *V. americana* (French and Moore 2003), and *P. maackianus* (Ni 2001). In plants and algae, photo-acclimation to low-light conditions normally involves increased synthesis of light-harvesting chlorophyll proteins for photosystems to increase light harvesting efficiency (Anderson et al. 1995, Walters 2005). As a result, higher light harvesting efficiency increases apparent quantum yield as observed in *S. pectinata* in this study. Anderson et al. (1995) summarised that photosynthetic apparatus acclimated to low-light would have more chlorophyll-b, due to increases in chlorophyll-b associated light-harvesting complexes (Walters 2005), therefore lowering the chlorophyll-a: b ratio. The changes in chlorophyll-a to carotenoid ratios is a response in acclimation to high-light rather than to low-light, the decreases in chlorophyll-a to carotenoid ratios in photosynthetic organisms indicates better protection against high-light stress (Blindow et al. 2003, Gerhardt et al. 2009). The photo-acclimation in leaves of *S. pectinata* in this study agrees with the well-established understanding on photo-acclimation in algae and higher plants (Anderson et al. 1995, Walters 2005, Moejes et al. 2017).

Interestingly, despite low-light acclimated leaves showing increased chlorophyll concentration for light-harvesting efficiency in all scenarios, the associated physical adjustment in the photosynthetic apparatus might differ. In acclimation to low light, field plants demonstrated significantly lower chlorophyll-a: b ratios but limited increases in chlorophyll-a content, whereas the growth chamber plants showed higher chlorophyll-a content but limited decreases in chlorophyll-a: b ratios. Compared to the high light treatment, the low-light treatment in the growth chamber had reduced light intensity, while the low-light treatment in the lake differed in spectral quality and was in changing light conditions besides reduced light intensity. Further research on acclimation to spectral

quality and to changing light environments would be need to understand the different pigment adjustments in different scenarios in this study.

### ***Low Light Acclimations and Plant Survival in Turbid Water***

Morphologically, low light stressed *S. pectinata* produced longer stems and longer leaves to get closer or reach the water surface sooner, where light is not or less limiting. Longer leaves constructed with lower DW: FW ratios in LL plants also increased the photosynthetic leaf area per unit leaf dry matter. Physiologically, low-light acclimated leaves had higher apparent quantum yield. However, *S. pectinata* in the morphological experiment did not escape the low-light condition as they were under shading not in turbid waters. We suggest that insufficient energy started to interfere with the low light-induced acclimations, resulting in rapid senescence of leaves, constrained size of secondary shoots, less final biomass and fewer tubers.

In the turbid water column in Te Waihora (Lake Ellesmere), *S. pectinata* could escape the low-light condition via shoot elongation to reach full sunlight on the water surface in the shallow area. However, successful escape of the low-light condition would depend on water depth, initial tuber biomass, and temperature. Because Initial tuber weight of *S. pectinata* positively correlates with the height of grown plants (Vermaat and Hootsmans 1994b), and temperature positively correlates with the relative growth rate of *S. pectinata* (Vermaat and Hootsmans 1994a).

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### **Chapter 3: Leaf Demography and Photosynthesis of *Stuckenia pectinata* in direct and stepwise salt acclimation**

#### **Abstract**

In order to inform the re-establishment of a macrophyte bed (primarily *Stuckenia pectinata*) into Te Waihora (Lake Ellesmere), a brackish lagoon periodically open to the sea that had salinity levels fluctuating at  $8.4 \pm 3.8$  ppt (mean  $\pm$  standard deviation) and occasionally extremely high levels exceeding 25 ppt, growth performance of *S. pectinata* in response to different salinities (6, 12, and 20 ppt) was investigated. Further, the resilience of *S. pectinata* to a lethal salinity level and plant performance through both abrupt and stepwise salt acclimations was studied. Three experiments were designed accordingly. Leaf demography, leaf length, leaf photosynthetic pigments, and leaf photosynthesis-irradiance response were measured. *S. pectinata* grew and survived in salinities up to 12 ppt. Under salinity stress, *S. pectinata* had reduced leaf production rates, fewer leaves and similar or reduced leaf length with increasing salinities. As a result, plants displayed reduced photosynthetic leaf area, and consequently less overall carbon assimilation. Exposure to 20 ppt for a week caused irreversible shoot death, but after returning to freshwater, belowground parts were able to support the growth of new shoots. Through either stepwise acclimation or direct acclimation, the same degree of salinity tolerance was achieved, as evidenced by similar photosynthesis-irradiance responses and concentrations of photosynthetic pigments, but direct acclimation involved higher leaf senescence rates.

Key words:

*Stuckenia pectinata*, leaf production rate, photosynthetic leaf area, photosynthesis, resilience, direct and stepwise acclimation

## Introduction

Te Waihora (Lake Ellesmere) is a large, shallow, wind-swept lagoon located on the east coast of the South Island of New Zealand, and is super-trophic and highly turbid (Gerbeaux and Ward 1991, Lomax et al. 2015). The lake is periodically opened to the sea for water level management (Reeves 1990). The marginal area of this lake used to be the habitat of a prosperous submerged macrophyte bed (primarily *Stuckenia pectinata* and *Ruppia spp.*) until the “Wahino” storm that destroyed the macrophytes in 1968 (Gerbeaux 1993). Since this event, the submerged macrophytes have only rarely and sparsely been observed (Gerbeaux 1993, Jellyman et al. 2009). Because a submerged macrophyte bed would ecologically favour a clear water state in the lake marginal area (Scheffer et al. 1993, Jellyman et al. 2009) that is preferred by the local habitants, there is an aspiration to re-establish a macrophyte bed in Te Waihora (Lake Ellesmere) through transplantation of mature plants to chosen sites. *Stuckenia pectinata* is considered the most suitable indigenous species for transplantation due to its tolerance of turbidity (Gerbeaux and Ward 1991) and fluctuating salinity (Jellyman et al. 2009).

Besides biotic factors (phytoplankton and periphyton etc.), light availability and salinity are the two primary abiotic factors constraining the regeneration of macrophytes in the lake (Gerbeaux 1989). Low light availability challenges macrophyte re-establishment by diminishing its ecological niche: light determines the maximum colonisable depth (0.48 m) that is shallower than the minimum colonisable depth (2.1 m) set by wave actions (Gerbeaux and Ward 1991, Jellyman et al. 2009). With wave energy dampened by wave barriers, acclimation in *S. pectinata* to a low-light stressing environment becomes critical for the success of the transplantation, and this was studied in detail in *Chapter 2*.

Salinity strongly affects macrophyte occurrence in brackish aquatic systems (Hinojosa-Garro et al. 2008, Robertson and Funnell 2012). Salinity fluctuation in Te Waihora (Lake Ellesmere) in the first half of the 20<sup>th</sup> century may well explain the decline and recovery of macrophyte distribution and biomass (Gerbeaux 1993). The optimum salinity for the growth of *S. pectinata* is genetically dependent, and the best salinity for growth has been found to be 0 ppt for freshwater populations and 6 ppt for brackish populations (van Wijk et al. 1988). Previous studies suggested that *S. pectinata* could grow at salinity levels less than 10 ppt

and endure (with reductions in biomass) at salinities as high as 16 ppt (van Wijk et al. 1988, Shili et al. 2007, Borgnis and Boyer 2016). Being periodically open to the sea, salinity sampled monthly in Te Waihora (Lake Ellesmere) was between 5 ppt and 10 ppt most of the time (Gerbeaux 1989), but ranged from 2 ppt to 27 ppt (2010 to 2016 data from Environment Canterbury). Except for the extremely high salinity at 27 ppt, the effects of which would likely be devastating, the salinity range in the lake likely provides windows for the growth of *S. pectinata*.

Fundamentally, salinity causes osmotic stress in plant cells over the short-term, and ionic toxicity over the long term. Although the mechanism of ionic detoxification in *S. pectinata* has not been revealed yet, two types of detoxification processes, in general, have been reported for plants and algae: ion exclusion and ion compartmentation, parallel with the synthesis of organic compounds for osmo-regulation (Ralph 1998, Rout and Shaw 1998, Husain et al. 2003, Parida and Das 2005, James et al. 2006, Läuchli and Grattan 2007, Munns and Tester 2008, Pagter et al. 2009, Hagemann 2011, Pandolfi et al. 2012, Garrote-Moreno et al. 2015, Piro et al. 2015, Janda et al. 2016). If the detoxification processes fail, increasing sodium concentrations in the cytoplasm in plant cells can generate adverse effects in chloroplast composition, photosynthetic processes, and lead to leaf senescence (Parida and Das 2005, Läuchli and Grattan 2007, Munns and Tester 2008, Janda et al. 2016), all of which constrain carbon assimilation.

Previous studies focused on biomass accrual and the number of shoots of *S. pectinata* in response to stress (van Wijk et al. 1988, Hall et al. 1997, Shili et al. 2007, Borgnis and Boyer 2016). Salinity tolerance of *S. pectinata* is genetically determined, and freshwater populations grow best in fresh water while brackish populations grew best at low salinities (3 ppt) in terms of dry biomass production and number of shoots (van Wijk et al. 1988). Dry biomass production of *S. pectinata* is negatively related to salinity levels (up to 15 ppt) (Borgnis and Boyer 2016).

With regards to *S. pectinata* in Te Waihora with a light-limiting water column, how different salinity levels affect the characteristic associated with light utilisation (leaf production, leaf photosynthetic area, and leaf photosynthesis) are our concerns. We hypothesize that leaf production rates and photosynthesis decrease with increasing salinities until a lethal level,



beyond which plant dies. Because salinity in Te Waihora occasionally jump to above 25 ppt that is beyond all the salinities reported to support growth, we are curious about the resilience of *S. pectinata* in response to the time of exposure to a lethal salinity level. We hypothesize that there is a threshold time of exposure to a lethal salinity level, beyond which *S. pectinata* lost its resilience. In Te Waihora, salinity either increases rapidly due to lake opening or increase gradually due to evaporation or small incremental seawater inflows, e.g. wave overtopping of the barrier bar in storms. To account for these two scenarios, we test the plant performance via a direct versus a stepwise salinity increase to a target salinity level.

In this study, the aim was to assess: 1) the effects of different salinities on leaf production, photosynthetic leaf area, leaf photosynthesis in *S. pectinata*; 2) the resilience of *S. pectinata* to a lethal salinity level with different exposure time; 3) the difference in plant performance through direct versus stepwise salinity increases.

## **Material and Methods**

### ***Experimental Design***

The salinity effects on *S. pectinata*, plant resilience to lethal salinity level, and plant performance through direct and stepwise acclimation pathways were studied in three consecutive experiments. The first addressed the effect of salinity on *S. pectinata* after acclimation to a range of salinities, termed “salinity stress” experiment; the second addressed recovery in fresh water after different number of days of exposure to a lethal salinity level, named “resilience experiment”; and the third addressed whether stepwise increase in salinity allowed a different response compared to direct acclimation, named “stepwise versus direct acclimation” experiment.

Experimental material was obtained from a population of *S. pectinata* that originated from tubers collected in a tributary of Te Waihora. This population grew in a freshwater aquarium (Volume: 3.2 m<sup>3</sup>, Depth: 0.6 m) in greenhouse conditions. Each replicate plant was obtained as follows. A healthy shoot of 40 to 50 cm height was cut at the sediment-water interface, and its basal leaf was removed to expose an internode. The shoot was then re-planted with

the internode buried in sediment (fine sand and clay, volume ratios 2:1) in 150 mL, 8 cm tall plastic beakers that had a thin layer of sand on top of the sediment. The fine sand was collected from a beach at the east coast near Te Waihora (Lake Ellesmere) and the clay was sieved sediment collected at the Timber Yard Point of the lake. The replanted shoots were kept in the greenhouse. Three weeks later new rhizomes developed from the internode and sent up new shoots. The old shoot was removed and new shoots were used in the “salinity stress” experiment and the “resilience” experiment.

### Salinity Stress Experiment

*S. pectinata* were pre-acclimated to four salinity levels (0, 6, 12, and 20 ppt) for 20 days in a 20°C growth chamber. There were 5 replicates for each salinity treatment. The plants were in a 5 by 4 matrix, where the first 4 columns formed a Latin Square and the last column had each treatment randomly assigned, in such a way to minimise the random error associated with space differentiation in the growth chamber. The medium comprised tap water (containing 0.10 - 0.13 mg /L phosphate; 1.0 - 1.1 mg /L nitrate-nitrogen) and artificial sea salt (Instant Ocean®, USA). Every plant was kept in individual 4L (22 cm deep) medium in a white plastic container. The irradiance at the water surface varied between 80 and 100  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  (Halogen light source) and the day length was 12 hours. Every container was bubbled with ambient air continuously at a rate that did not disturb the positions of leaves in the water column. pH was monitored twice a week for each container and the water was replaced for all plants if pH reached 8.5 or above in any container. Otherwise, water was replaced every 3 weeks. Water was adjusted to 20°C before replacements. The water level was maintained by adding tap water every 2 days to counterbalance evaporation. Evaporation amounted to less than 1% of the water volume over the 20-day period. At the end of the pre-acclimation period, every plant was trimmed such that only one shoot, all of a similar size, were left. The experiment then continued for another 24 days. On day 1, 8, 16, and 24, the number of green and senesced leaves were counted, the total length of green leaves was measured for each plant. The dead leaf proportion on each sampling day, and leaf production rate and senescing rate between sampling days were calculated. Average green leaf length was calculated as total green leaf length divided by the number of green leaves. On day 22, one healthy leaf per plant was

excised for photosynthesis-irradiance curves analysis, and measured for fresh and dry weight. Fresh weight (FW) of each leaf was measured after rinsing with tap water and blotting dry with tissue paper. Leaf dry weight (DW) was measured after drying for 24 hours at 80 °C. Dry weight to fresh weight (DW: FW) ratio was calculated for each leaf. On day 23, another healthy leaf from each plant was excised for photosynthetic pigments analysis. On day 24, the remaining aboveground plant parts were harvested, washed and analysed for dry weight (DW).

### The Resilience Experiment

Previously greenhouse propagated *S. pectinata* were kept in exactly the same conditions as described in the “Salinity Stress” experiment. Fifteen plants were kept in 0 ppt for two weeks. In the third week, five replicates per group and three groups in total were exposed to 20 ppt for 7 days, 3 days, and 0 days. The treatment replicates were arranged in a 5 by 3 matrix, where the three treatments alternated along row by row, such that any 3 by 3 matrix within forms a Latin Square design. The “3 days in 20 ppt” group was exposed to 20 ppt 4 days after the “7 days in 20 ppt” group, so that treatments terminated at the same time. The control group “0 days in 20 ppt” was always in fresh water. Artificial sea salt (Instant Ocean®, USA) was again used for salinity adjustment. At the end of the 3<sup>rd</sup> week, the medium surrounding all plants was replaced with fresh water, and every plant was trimmed to leave one shoot of similar sizes. These shoots are called “old shoot”. The experiment continued for another 19 days, and the growth of the old shoot and production of “new shoots” was followed. On day 1, 7, 13, and 19, the number of green leaves and senesced leaves were counted, separately for the “old shoots” and the “new shoots”, and the proportion of senesced leaves was calculated.

### Stepwise Versus Direct Acclimation Experiment

Field collected *S. pectinata* tubers ( $44 \pm 6$  mg, mean  $\pm$  standard error) were planted in sediment (fine sand and clay, volume ratios 2:1) in 150 mL, 8 cm tall plastic beakers in 25 cm deep tap water under  $50 \mu\text{mol photons/m}^2/\text{s}$  (Halogen Light, 12 h : 12h day-night cycles) at 20 °C in growth chamber conditions. After four weeks, sixteen well-growing plants of similar

sizes were chosen and each kept in 0 ppt water in a 22 cm deep, 4L plastic container. The irradiance at the water surface varied between 70 and 90  $\mu\text{mol photons/ m}^2 \text{ /s}$  (Halogen light) and day-night cycle was 12 to 12 hours. After another two-weeks, all plants were exposed to 6 ppt saline water. After 4 days in 6 ppt, salinity for eight plants was raised to 12 ppt by adding artificial sea salt and the experiment continued for another 48 days. The plants in the two salinity treatments were arranged in a 4 by 4 matrix, within which any 2 by 2 sub-matrix formed a Latin square design. Water management was as described in the “Salinity Stress” experiment. On day 0, 4, 8, 20, 32, and 40, the number of green leaves and senesced leaves were counted separately, and the proportion of senesced leaf was calculated. The total length of green leaves was estimated and the average leaf length was calculated. On day 38 and day 39, one healthy leaf per plant was excised on each day for photosynthesis-irradiance curve analysis and photosynthetic pigment analysis respectively. On day 48, plants were harvested, washed in tap water, and separated into green leaves, senesced leaves, stems, rhizomes and roots, and tubers. All these parts were analysed for FW and DW, and the DW: FW ratios was calculated for each plant. Total aboveground and belowground dry weight also was determined.

As a comparison to the stepwise acclimation, direct acclimation was investigated by following the performance of freshwater grown plants after abrupt exposure to salinities at 6 ppt, 12 ppt, and 20 ppt. The plant material and experiment setup were the same as in the “Salinity Stress” experiment. This direct acclimation experiment investigated the same plants in the “Salinity Stress” experiment during the 20 days pre-acclimation period. During these 20 days, the number of green leaves and senesced leaves were recorded separately, and calculated for leaf production rate, leaf senescing rate, and dead leaf proportions on day 0, 6, and 12.

### ***Total Green Leaf Length Estimation and Photosynthetic Pigment Analysis***

*S. pectinata* was flattened on a gridded board that was 5 mm below the water surface, and a photo was taken with a camera (Leica X2) from 50 cm above the water surface. The photo was orthogonally transformed (Adobe Lightroom 5) then analysed (Image J) for total green leaf length for each plant.

Pigments were analysed within an hour after leaf collection. Each leaf was ground with a mortar and a pestle in 5 mL 90% acetone for 30 seconds, before the extracts were transferred into a 15 mL plastic vial. This step was repeated twice to ensure a complete extraction. After storing at 6°C for 24 hours, the extracts were centrifuged at 3000 r.p.m for 15 minutes. The absorbance of the supernatant was then measured at 470 nm, 647nm, 664nm, and 750nm (Spectrophotometer D3900, Hach, USA) in a 1 cm glass cuvette. Chlorophyll-a and chlorophyll-b concentrations were calculated according to Porra (2002) and total carotenoid concentration was estimated according to Wellburn (1994). Chlorophyll-a:b ratio and chlorophyll-a to total carotenoid (Chl-a: Caro) ratio was calculated.

### ***Photosynthesis-Irradiance (PI) Curve Analysis***

For each leaf collected, oxygen production rates were measured in the dark and under a gradient of PARs (Table 1). Darkness was created by wrapping samples with aluminium foil. Irradiance was provided by three, radially arranged 100 W LED floodlights (colour temperature 4000 K) placed 50 cm above a temperature-controlled water bath. The different PARs were created using different layers of white translucent cloth placed 30 cm below the LED lights, and a LiCor Li-192 quantum sensor was used to measure PAR. The gradient of PARs achieved was 0, 21, 46, 66, 89, 129, 192, 242, and 317  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . Two polycarbonate boards were placed in-between white cloth to increase light reflections and scattering as the light pass through, therefore the light field was more homogeneous (Table 1) for plant incubations in the water bath.

Each element in the water bath (the water bath container, the water pump, the heating unit, and the rack) was in black colour to prevent reflections and to ensure that incubations only received PAR from the filtered LED light. A circular area, 23 cm in diameter at the surface of the water bath, was illuminated almost homogeneously (Table 1) and could accommodate up to 22 incubation glass vials on a rack 1 cm below the water surface. The water bath was maintained between 20.4 and 20.8 °C and circulated using a submersible pump (23 w, 1001, EHEIM, GmbH).

Incubations were of a single leaf sealed in a 12.2 mL glass vial filled with air-saturated tap water adjusted to the treatment salinities of leaf samples with artificial sea salt, and

enriched with 10 mmol/L bicarbonate to prevent carbon depletion affecting the photosynthetic rate. Three blank glass vials as controls were incubated at each time along with leaf samples. Two 5 mm diameter glass beads were placed in each vial to assist in vial water mixing. At the end of incubation under each light level, each glass vial was mixed by inverting several times, opened and a 1 ml volume of incubation medium withdrawn using a syringe into a Presens FTCM flow-through sensor and dissolved oxygen was measured using a PreSens Microx 4 control unit (PreSens, GmbH, Germany). Oxygen evolution or uptake was determined as the difference from the controls, normalised to time and to the amount of chlorophyll-a. Incubations were first in darkness, after which the leaves were incubated incrementally over a gradient of irradiance, with the incubation time determined by the irradiance treatment (Table 1).

Table.1 PAR treatments and incubation times used for determination of photosynthesis-irradiance relationships. (For each PAR treatment, incubation area PAR is presented with mean and range)

Light Level	1	2	3	4	5	6	7	8
Mode ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )	21	46	66	89	129	192	242	317
Range ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )	2.6	3.6	1.8	1.8	3.0	3.0	9.0	14.0
Incubation time (s)	3600	3600	2700	2700	1800	1800	1800	1800

After all incubations were complete, leaves were dried at 80 °C for 24 hours, cooled and their DW were determined. For each leaf, oxygen production rate at each light level was calculated as the rate of change of oxygen concentration, per unit chlorophyll-a, yielding units of  $\mu\text{g O}_2 \text{ mg}^{-1} \text{ Chl-a s}^{-1}$ . Photosynthetic rates against irradiance were fitted with Hyperbolic Tangent Model (Jassby and Platt 1976) for each salinity treatment for presentation purpose.

Hyperbolic Tangent Model:

$$P = P_{\max} * \tanh(\alpha I / P_{\max}) + R \quad (\text{Equation 1})$$

Where:

P: net photosynthetic rate, as a dependent variable

I: photosynthetic active radiation, as an independent variable

$\alpha$ : apparent quantum yield, the slope of the curve at low irradiance before the onset of maximum photosynthesis

$P_{\max}$ : light-saturated (maximum gross) photosynthetic rate

$R$ : dark respiration rate

Hyperbolic Tangent Models were fitted with the Analytic Gauss-Newton regression algorithm (JMP 9, SAS®), and the three key parameters ( $\alpha$ ,  $P_{\max}$ , and  $R$ ) were estimated simultaneously.

### ***Data Analysis***

The number of green leaves, dead leaf proportion, leaf production rate, and leaf senescence rate were analysed with repeated measures ANOVA when normality of residuals from the ANOVA linear fit was confirmed with Shapiro-Wilk test. Post-contrast test (Student t test) using least square means from the ANOVA fit was employed to further detect differences between treatments by time or among sampling times by treatment. Data was transformed if the distribution of residuals from repeated measure ANOVA fit was not normal. In the “Salinity Stress” experiment, the number of green leaves was square root transformed and the leaf production rate was  $\log_{(10)}$  transformed. In the stepwise acclimation experiment, leaf senescence rate was square root transformed.

In the resilience experiment, the normality of residuals could not be met for green leaf number and dead leaf proportions after data transformations. Therefore, multivariate analysis of variance (MANVOA) was used to analyse treatment effects on dead leaf proportions and green leaf number of the new shoots. For the old shoots, dead leaf proportion on day 1 was analysed using ANOVA, followed by post-Tukey HSD test, and dead leaf proportion and green leaf number for the control group were analysed using repeated measures ANOVA. For the new shoots, green leaf number of the new shoot on day 19 were analysed using ANOVA. In the direct acclimation experiment, leaf senescence rates against all salinity levels was analysed using linear regression.

Plant biomass, pigments concentrations and ratios, photosynthetic rates under each actinic light level, and DW: FW ratios were compared using one-way ANOVA between treatments. All statistical analysis was performed using JMP 9 (SAS®, JMP® Version 9, SAS Institute Inc.).

## Results

### *Leaf Demography and Biomass*

#### Salinity Stress

Four out of five replicates in the 20 ppt group had all the aboveground tissue died before day 1, and one replicate had 4 green leaves from new emerging shoots on day 1 and reduced to 2 leaves through the experiment. The 20 ppt group was considered a lethal level for *S. pectinata* and is not included in Figure 1. The number of green leaves was affected by salinity treatments (repeated measures ANOVA,  $p < 0.001$ ) and time ( $p < 0.001$ ), as well as time and treatment interactions ( $p < 0.001$ ). The 0 ppt group had the highest number of green leaves and the 12 ppt group had the lowest green leaf number (Figure 1a). For the 12 ppt group, the number of green leaves did not change with time, while for the 0 ppt and 6 ppt groups, the number of green leaves increased with time. The number of leaves increased from day 1 to day 24 for the 0 ppt group, and increased from day 1 to day 8 for the 6 ppt group (Figure 1a).

Consistently, significantly higher leaf production rate than leaf senescence rate were observed in 0 ppt plants through the experiment (repeated measures ANOVA,  $p = 0.002$ ), and in 6 ppt plants from day 1 to day 16 but not after day 16 (Contrast test using least square means), whereas in 12 ppt plants no difference was observed between leaf production rate and senescence rate (repeated measures ANOVA,  $p = 1.0$ ) (Figure 1c). The leaf production rate was negatively correlated with increasing salinities (repeated measures ANOVA,  $p < 0.01$ ), and was not affected by time ( $p = 0.28$ ) or by time and treatment interactions ( $p = 0.054$ ). Leaf senescence rate was only affected by treatments (repeated measures ANOVA,  $p < 0.01$ ), and was higher at 6 ppt than at 0 ppt and 12 ppt, where the latter two did not differ (Figure 1c).



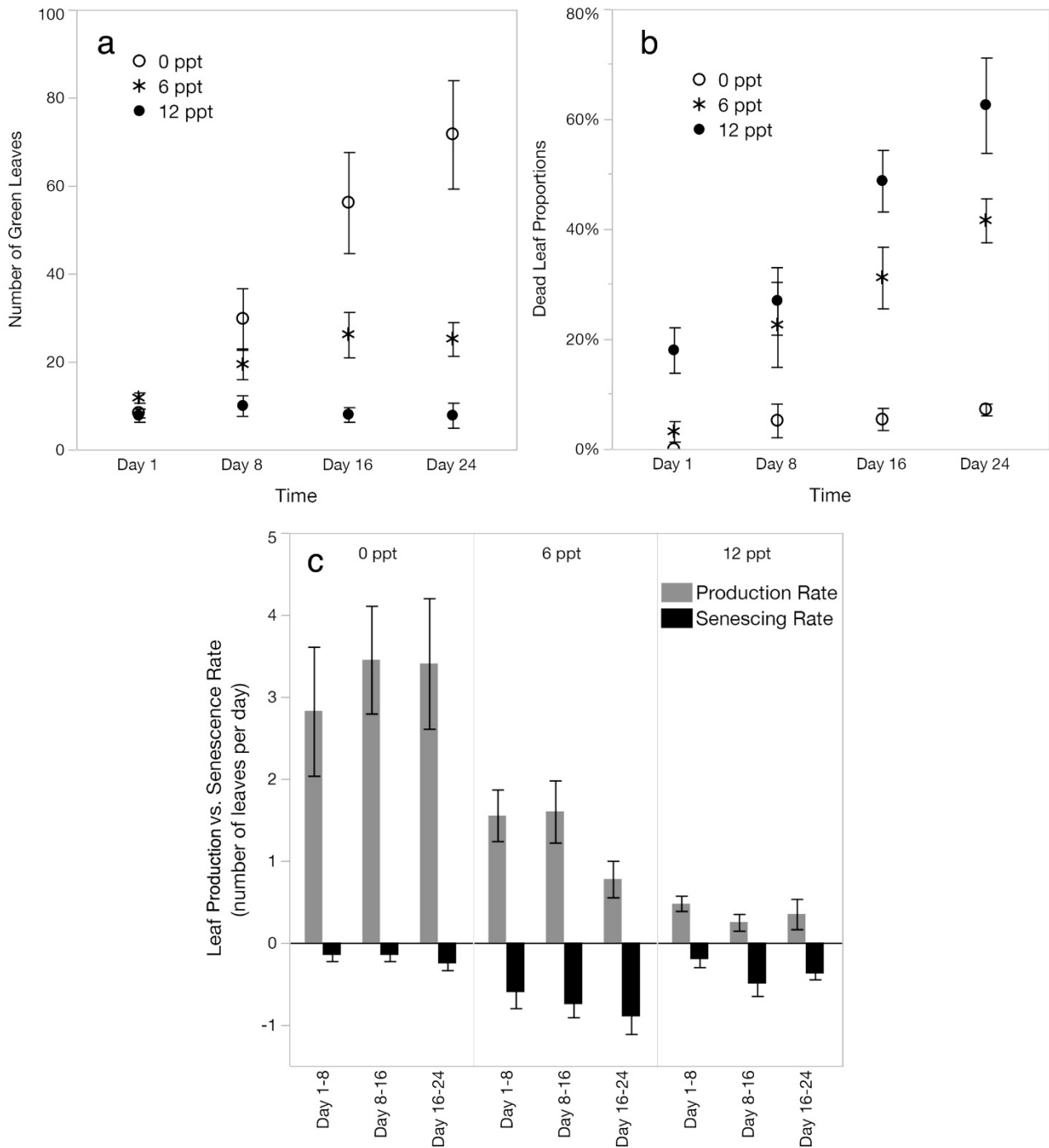


Figure 1. Leaf dynamics of *S. pectinata* after acclimation to salinities at 6 ppt, 12 ppt, and 20 ppt: a) the number of green leaves; b) the number of dead leaves divided by total number of leaves; c) leaf production rates and leaf senescing rates (figures were produced from raw data that are presented as mean plus/minus standard error of the mean)

The proportion of dead leaves was affected by salinity (repeated measures ANOVA,  $p < 0.001$ ), time ( $p < 0.001$ ), and their interactions ( $p < 0.001$ ). The 12 ppt group had the highest dead leaf proportion and the 0 ppt group had the lowest (Figure 1b). The 12 ppt group had

higher proportions of dead leaves on day 1 than the 0 ppt group and the 6 ppt group. (ANOVA,  $p = 0.001$ ). For the 0 ppt group, the dead leaf proportion did not change with time, while dead leaf proportions increased with time for the 6 and 12 ppt groups.

The average length of green leaves was affected treatment (repeated measures ANOVA,  $p < 0.001$ ) and time ( $p = 0.001$ ) but not by time and treatment interaction ( $p = 0.054$ ). Leaves were shorter on average for the 12 ppt plants than the 0 ppt and 6 ppt plants, while the latter two were not significantly different. Leaf length decreased over time for all treatments (Figure 2).

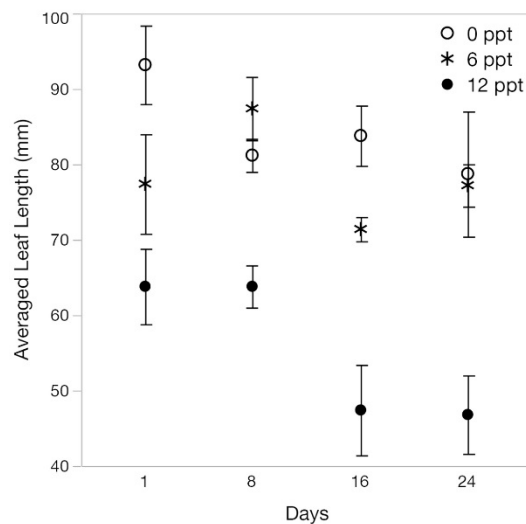


Figure 2. Averaged leaf length over time per treatment

The final aboveground biomass was  $0.10 \pm 0.01$  g DW for the 0 ppt group,  $0.05 \pm 0.01$  g DW for the 6 ppt group, and  $0.01 \pm 0.01$  g DW for the 12 ppt group. The 0 ppt group had the highest aboveground biomass and the 12 ppt group had the lowest.

## Resilience Experiment

For the old shoots, dead leaf proportions were different on day 1 (ANOVA,  $p < 0.001$ ): the “0 days in 20 ppt” group had the lowest dead leaf proportions and the “7 days in 20 ppt” group had the highest (Figure 3c). After returning to freshwater, leaf senescence continued and the “7 days in 20 ppt” group had all leaves senesced on day 7, the “3 days in 20 ppt” gradually increased its senesced leaf proportion and on day 13 four out of the five replicates

had senesced all leaves. The dead leaf proportions of the control group did not change with time (repeated measures ANOVA,  $p = 0.44$ ). Consistent with leaf senescence, the number of green leaves decreased for old shoots in the “3 days in 20 ppt” and “7 days in 20 ppt” groups, while green leaf number increased for old shoots in the control group (repeated measures ANOVA,  $p = 0.04$ ) (Figure 3a).

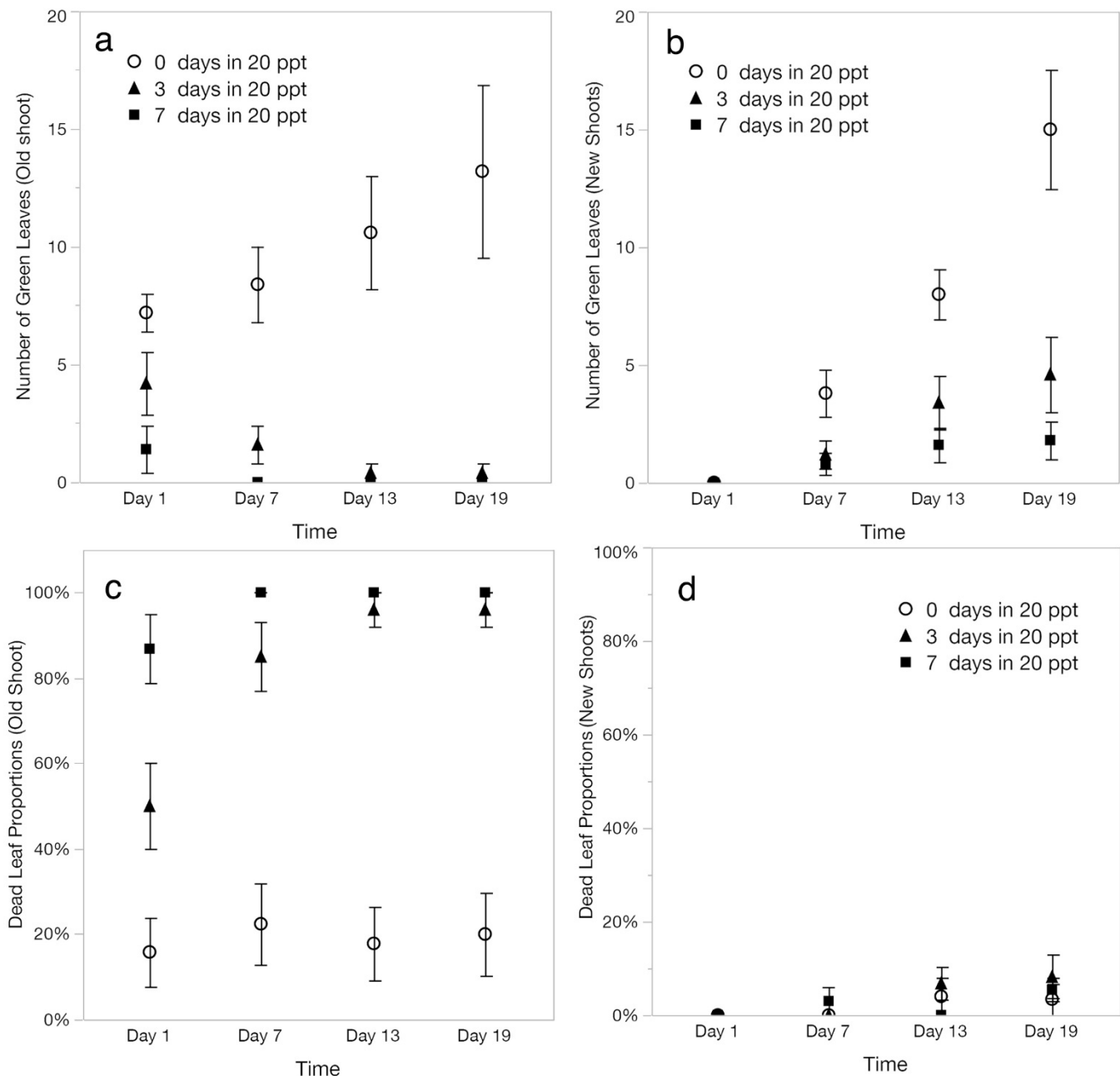


Figure 3. The number of green leaves and proportions of senesced leaves over time (old shoot was the shoot existing on day 1; new shoots were shoots sprouted after day 1; Values presented as mean  $\pm$  standard error).

For the newly developed shoots, leaf senescence was not affected by the prior salinity treatments (MANOVA,  $p = 0.7$ ) (Figure 3d). The number of green leaves on the new shoots, however, differed among treatments (MANOVA,  $p < 0.001$ ), and on day 19 the control group had more green leaves than the “3 days in 20 ppt” and the “7 days in 20 ppt” group, while the latter two did not differ significantly (Tukey HSD test) (Figure 3b).

### Stepwise Acclimation versus Direct Acclimation

In the stepwise acclimation experiment, the number of green leaves was affected by treatment (repeated measures ANOVA,  $p = 0.03$ ) and time ( $p < 0.001$ ), but not by their interaction ( $p = 0.11$ ). The 6 ppt plants had more green leaves than the 12 ppt group (Figure 4a). The number of green leaves increased from day 1 to day 20, reached its maximum between day 20 and day 32, and decreased after day 32 (Tukey HSD test using least square mean) (Figure 4a).

Within each treatment, from day 0 to day 20 leaf production rate was greater than leaf senescence rate for plants in both 6 ppt (repeated measures ANOVA,  $p < 0.001$ ) and 12 ppt ( $p = 0.002$ ); from day 20 to day 40, greater leaf senescence rate than leaf production rate was observed in 6 ppt plants (repeated measures ANOVA,  $p = 0.01$ ), whereas senescence rate was not significantly greater than leaf production rate in 12 ppt plants (repeated measures ANOVA,  $p = 0.17$ ).

Between treatments, leaf production rate was affected by treatment (repeated measures ANOVA,  $p = 0.01$ ) and time ( $p = 0.01$ ), but not by treatment and time interaction ( $p = 0.44$ ) (Figure 4c). Leaf production rate was higher in 6 ppt than in 12 ppt, stable before day 20, and gradually decreased between day 20 and 40 (Contrast test using group least square mean). Leaf senescence rate was only affected by time: it remained stable between day 0 and day 20, and increased from day 20 to day 40 (Contrast test using group least square mean).

The proportion of dead leaves demonstrated a continuous increase from day 0 to day 40 (repeated measures ANOVA,  $p < 0.001$ ), though there was no effect of salinity ( $p = 0.74$ ) or an effect of salinity and time interaction ( $p = 0.53$ ) (Figure 4b).

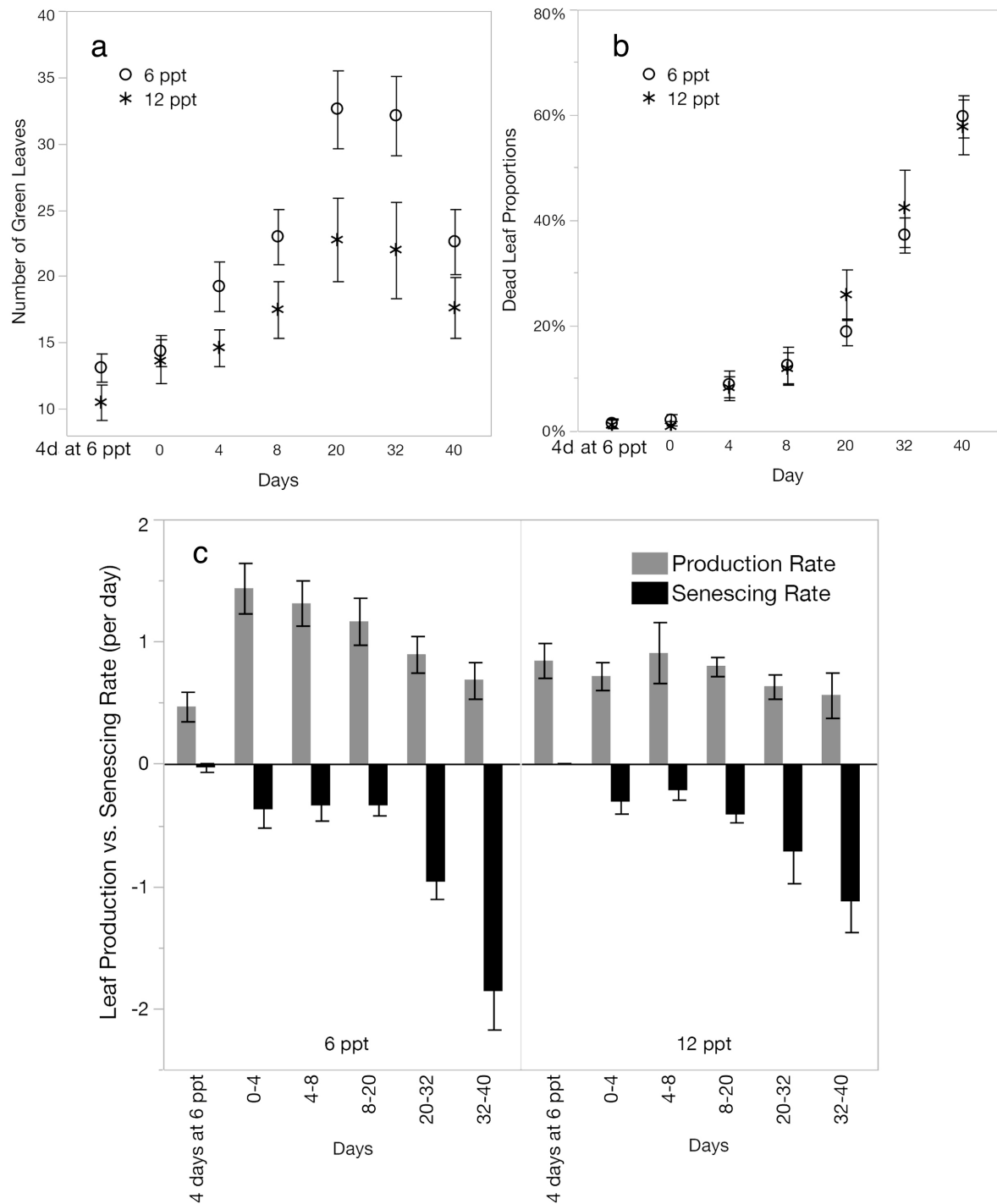


Figure 4. Leaf dynamics of *S. pectinata* after stepwise acclimation to 12 ppt vs. 6 ppt: a) the number of green leaves; b) the number of dead leaves divided by total leaf number; c) leaf production rate and leaf senescence rate. Values are presented as mean plus/minus standard error of the mean.

The average leaf length showed a continuous gradual decrease over time (repeated measures ANOVA,  $p < 0.001$ ) (Figure 5), but was independent of treatments ( $p = 0.22$ ) and treatment time interactions ( $p = 0.86$ ).

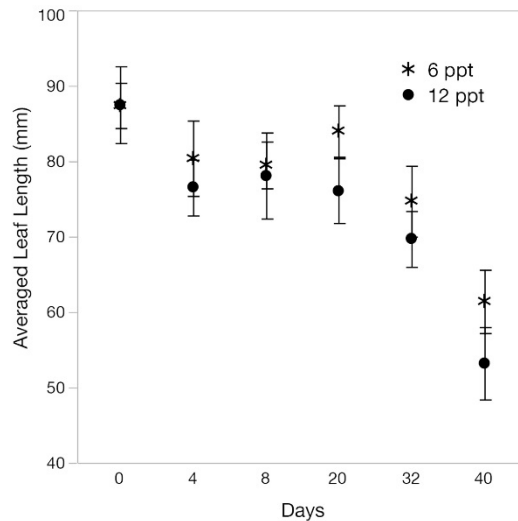


Figure 5. Averaged leaf length over time

On day 48, the total dry weight of aboveground parts did not differ between the groups (ANOVA,  $p = 0.17$ ) and was  $0.10 \pm 0.01$  g for the 6 ppt group and  $0.08 \pm 0.02$  g for the 12 ppt. Neither did the dry weight of belowground parts differ (ANOVA,  $p = 0.21$ ), and were  $0.09 \pm 0.01$  for the 6 ppt group and  $0.07 \pm 0.01$  for the 12 ppt group.

In the direct acclimation experiment, leaf production rate was higher for plants in 6 ppt than in 12 ppt (repeated measures ANOVA,  $p = 0.02$ ), increased with time ( $p = 0.005$ ), and was affected interactively by salinity and time ( $p = 0.03$ ): production rate between day 6-12 was higher than day 1-6 at 6 ppt, and was not different between day 1-6 and day 6-12 at 12 ppt (Contrast test using least square means) (Figure 6c). Leaf senescence rate was not affected by treatments (repeated measures ANOVA,  $p = 0.14$ ) or by time ( $p = 0.75$ ), nor by their interaction ( $p = 0.19$ ). However, upon the initial salinity exposure between day 1-6, there was a clear linear relationship between salinity and leaf senescence rates (linear regression analysis,  $p < 0.001$ ), which disappeared between day 6-12 (linear regression analysis,  $p = 0.06$ ) (Figure 6c). Upon the initial salinity exposure, 12 ppt salinity induced a higher leaf senescence rate than 6 ppt. The combined effects of less leaf production rate and higher initial leaf senescence rates at 12 ppt than at 6 ppt, contributed to lower leaf numbers

(repeated measures ANOVA,  $p = 0.02$ ) and higher dead leaf proportions on day 12 (Contrast test using least square mean in repeated measure ANOVA,  $p = 0.01$ ) (Figure 6 a, b).

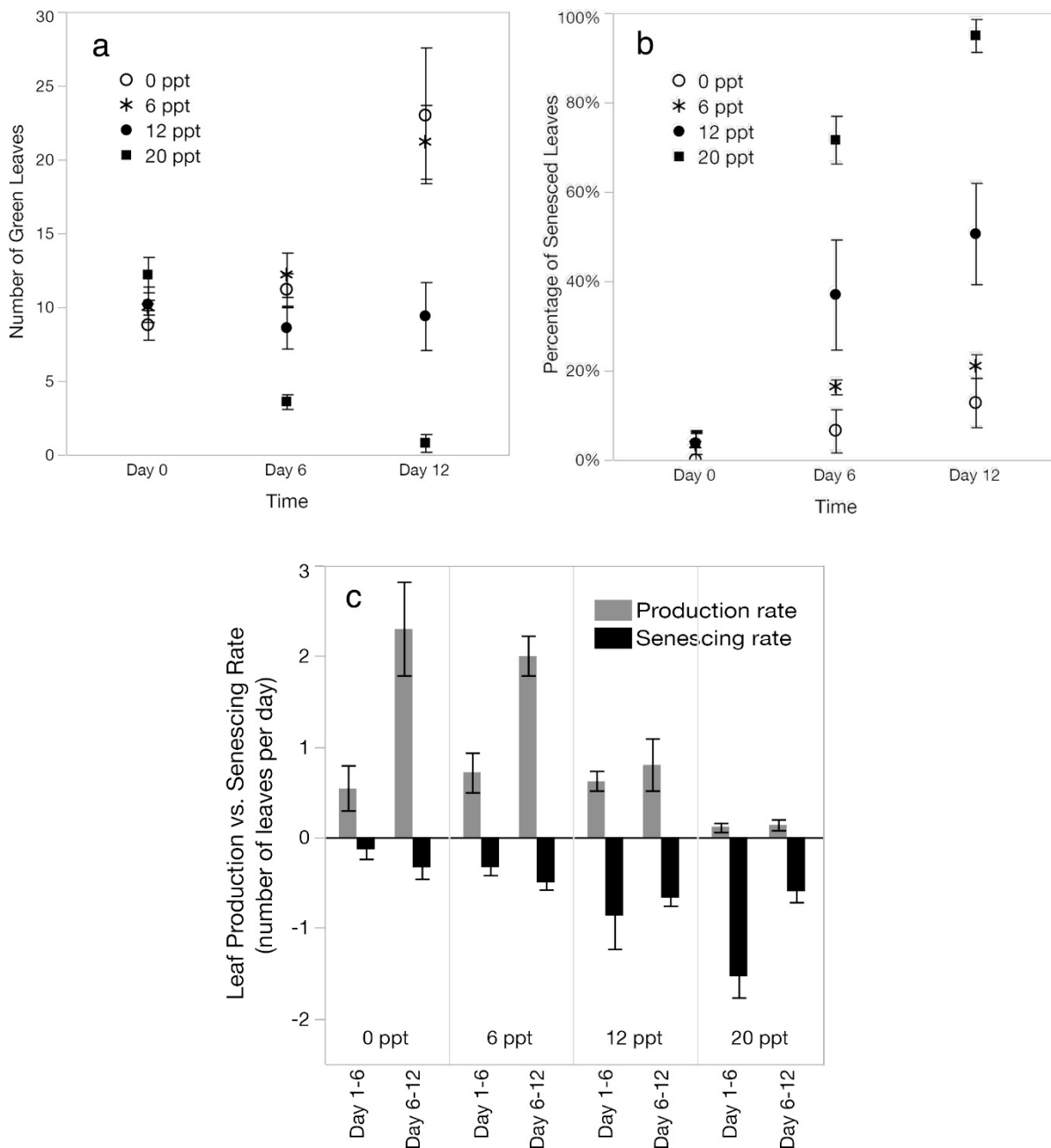


Figure 6. Leaf dynamics of freshwater *S. pectinata* after abrupt exposure to salinities at 6 ppt, 12 ppt and 20 ppt: a) the number of green leaves; b) the number of dead leaves divided by total leaf number; c) leaf production rates and leaf senescing rates. Values are presented as mean plus/minus standard error of the mean.

### ***Photosynthetic pigments, Photosynthesis and Dry Content***

In both experiments, there was no difference in chlorophyll-a concentrations between/among the treatments (Table 2). However, in the “Salinity Stress” experiment, the 0 ppt group had lower chlorophyll-a: b ratios and higher chlorophyll-a: carotenoid ratios than the 12 ppt group (Table 2).

Table 2. Pigment concentrations and ratios in green leaves. Data are presented as mean  $\pm$  standard error, different capital letter indicates significant difference.

	Treatment	Chlorophyll-a (mg /g DW)	Chlorophyll-a:b ratio	Chl-a:Caro Ratios
Salinity	0 ppt	19.9 $\pm$ 2.4	2.53 $\pm$ 0.04 <sup>A</sup>	4.4 $\pm$ 0.2 <sup>A</sup>
Stress	6 ppt	21.7 $\pm$ 0.8	2.55 $\pm$ 0.04 <sup>AB</sup>	4.2 $\pm$ 0.1 <sup>AB</sup>
	12 ppt	16.7 $\pm$ 1.7	2.66 $\pm$ 0.03 <sup>B</sup>	3.8 $\pm$ 0.1 <sup>B</sup>
Stepwise	6 ppt	12.6 $\pm$ 1.7	2.28 $\pm$ 0.03	3.3 $\pm$ 0.3
Acclimation	12 ppt	16.2 $\pm$ 1.3	2.26 $\pm$ 0.04	3.4 $\pm$ 0.1

Photosynthetic rate per unit chlorophyll-a was not significantly different at any actinic light level between leaves in 0 ppt, 6 ppt and 12 ppt in the “Salinity Stress” experiment, nor was there any difference between leaves in 6 ppt and 12 ppt in the “Stepwise Acclimation” experiment (Figure 7).

At higher salinities, DW: FW ratios were higher for all aboveground tissues, including green leaves, dead leaves, and stems, whereas DW: FW ratios of the belowground parts (rhizomes and roots) did not differ between treatments (Table 3 and Table 4).



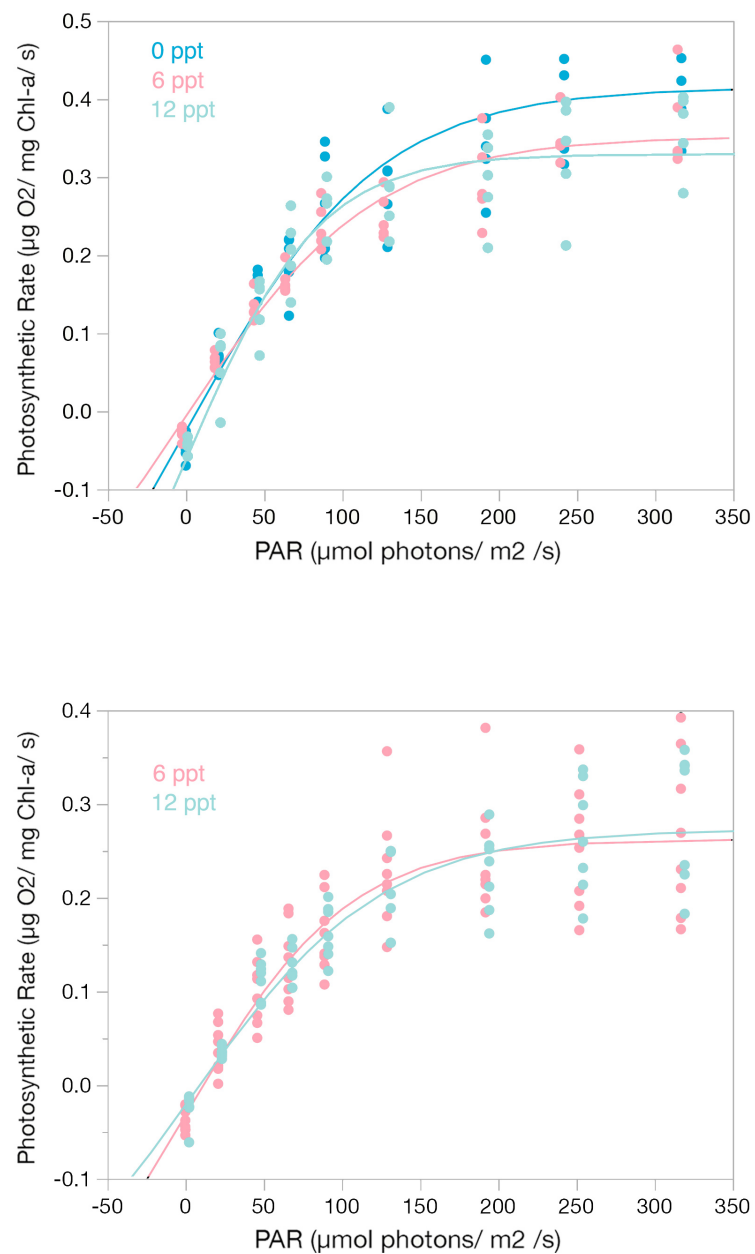


Figure 7. Photosynthesis-irradiance curves for the “Salinity Stress” experiment (the upper panel) and the “Stepwise Acclimation” experiment (the bottom panel)

Table 3. DW: FW ratios for leaves grown in “Salinity Stress” experiment. Data are presented as mean  $\pm$  standard error and significant level are indicated by different capital letters.

Treatment	0 ppt	6 ppt	12 ppt
Leaf DW:FW ratios	$7.6 \pm 1.0 \%^{AB}$	$7.3 \pm 0.3 \%^B$	$10.1 \pm 0.6 \%^A$

Table 4. DW: FW ratios for various plant tissues in the stepwise acclimation experiment. Data are presented as mean  $\pm$  standard error and significant level are:  $p$  - value  $< 0.05$  indicated by “\*”,  $p$  - value  $< 0.01$  indicated by “\*\*”;  $p$  - value  $< 0.001$  indicated by “\*\*\*”)

DW: FW ratios	6 ppt	12 ppt
Green Leaves	12.9 $\pm$ 0.3 % ***	15.8 $\pm$ 0.6 %
Senesced Leaves	9.3 $\pm$ 0.2 % **	10.4 $\pm$ 0.3 %
Stems	14.4 $\pm$ 2.0 % *	20.9 $\pm$ 1.0 %
Root and Rhizomes	16.9 $\pm$ 0.8 %	19.0 $\pm$ 1.2 %

## Discussion

### *Leaf Production and Senescence*

In acclimatised plants, leaf production rate was negatively related to salinity (Figure 1). Reduced leaf production rate in salinity stress has been reported for the non-halophyte submerged macrophyte *Potamogeton tricarinatus* (Warwick and Bailey 1998), and halophyte species, red mangrove *R. mucronata* (Hoppe-Speer et al. 2011) and black mangrove *Avicennia germinans* L. (Suarez and Medina 2005). Warwick and Bailey (1998) discussed that decline in leaf production in *P. tricarinatus* may result from increased senescence rates of older leaves, correspondingly less leaves for photosynthesis, and consequently less photosynthetic carbohydrate. van den Brink and van der Velde (1993) observed salinity reduced cell expansion and photosynthetic leaf area in *Potamogeton lucens* L., *Potamogeton perfoliatus* L., and *Potamogeton nodosus*. In non-halophyte species, leaf production rates could be limited by energy supply from photosynthesis due to accelerated shedding of older leaves and, therefore, less leaf photosynthetic area (Munns and Termaat 1986). Whereas in halophytes species, Hoppe-Speer et al. (2011) and Suarez and Medina (2005) attributed the constrained leaf production rates to a diversion of energy investment from development to the operation of salt tolerant mechanisms, as osmoregulation is costly. *S. pectinata* demonstrated enhanced leaf senescence and less photosynthetic area with increasing salinity levels in this study, and van Wijk et al. (1988)

mentioned *S. pectinata* accumulated glutamic acid with increased salinity, as a osmo-regulation strategy. Therefore, reduction in leaf production in salt stressed *S. pectinata* could be explained by less photosynthetic carbohydrate assimilated in combination a diversion of energy for osmo-regulation.

In acclimatised plants, leaf senescence rate increased for *S. pectinata* under salinity stress, as was higher for plants at 6 ppt than in 0 ppt (Figure 1). Although similar leaf senescence rate was observed in 12 ppt as in 0 ppt, the number of leaves was much smaller for the 12 ppt plants. The smaller leaf number likely confounds the effect of 12 ppt salinity on leaf senescence. Enhanced leaf senescence has been observed for other aquatic species. Necrosis occurred in *Callitriche stagnalis* when salinity increases from 0-2 ppt to 5 ppt in a week (Hinojosa-Garro et al. 2008). Jampeetong and Brix (2009) observed marked injury of old leaves in *Salvinia natans* one week after gradual salinity elevation from 0 to 5.8 ppt NaCl salinity or above. The leaves of *S. natans* became yellow-green then turned brown (Jampeetong and Brix 2009), quite similar to what we observed in *S. pectinata* in this study. Disturbed  $K^+$  acquisition resulting in high  $Na^+ : K^+$  ratio in the cytoplasm in leaf cells of *S. natans* is considered to be the reason, as external high  $Na^+$  concentration compete with  $K^+$  uptake and lead to internal  $K^+$  deficiency (Jampeetong and Brix 2009). Leaf senescence in *Potamogeton tricarinatus* upon salinity exposure was also caused by a high  $Na^+ : K^+$  ratio in leaves, and *P. tricarinatus* started to lose control over in-leaf  $Na^+ : K^+$  ratio at salinity above 2 ppt (Warwick and Bailey 1997). Studies on submerged aquatic angiosperms revealed that salt tolerant *Najas gramenia* was able to maintain intracellular  $K^+$  concentrations while salt sensitive *Najas indica*, *Hydrilla verticillata* had reduced intracellular  $K^+$  concentrations under sea salt stress, despite all of them having increased  $Na^+$  concentrations (Rout and Shaw 2001). Enhanced  $Na^+ : K^+$  ratio causes increased leaf senescence in *S. natans*, *P. tricarinatus*, *N. indica*, and *H. verticillata*. *S. pectinata* obtains  $K^+$  and  $Na^+$  directly from water (Huebert and Gorham 1983) and accumulated higher or equal concentrations of  $K^+$  than  $Na^+$  from the habitat, where concentrations of  $K^+$  were at least 10 times lower than concentrations of  $Na^+$  (van Wijk 1989). Given this, it would be more difficult for *S. pectinata* to accumulate  $K^+$  in a environment with elevated NaCl salinity to maintain a healthy  $Na^+ : K^+$  ratio. Increased  $Na^+ : K^+$  ratio in NaCl salinity likely induces leaf senescence in *S. pectinata* in this study.

### **Photosynthetic Leaf Area**

With increasing salinities, slower leaf production rates with enhanced leaf senescence rate in *S. pectinata* resulted in fewer number of green leaves (Figure 1). In the stepwise acclimation experiment, *S. pectinata* in 12 ppt also had fewer green leaves than in 6 ppt due to slower leaf production rates (Figure 4). In the “Salinity Stress” experiment, leaf length of the 12 ppt group became shorter than the 0 ppt and 6 ppt groups. As the leaf width was shown to be not dependent on salinities in a pilot experiment, leaf length indicates the photosynthetic leaf area for *S. pectinata*. Due to fewer leaves within increasing salinity levels and shorter leaves at salinity above 12 ppt, photosynthetic leaf area per plant decreases with increasing salinities. Reduced photosynthetic leaf area under salt stress in *P. lucens* L., *P. perfoliatus* L., *P. modosus* Dior (van den Brink and van der Velde 1993), and *S. natans* L. (Jampeetong and Brix 2009) all led to a reduced biomass production. The salt-induced leaf death, and consequently, reduced photosynthetic leaf area, and less carbonate to sustain growth have been reported for crops as reviewed in Munns (2002). Reduction in photosynthetic leaf area would synergistically stress the growth of *S. pectinata* in Te Waihora, where light interception is specifically important in low-light water column.

### **Pigments and Photosynthesis**

Despite the senescence of older leaves, chlorophyll-a per unit dry weight in green leaves and photosynthetic rates per unit chlorophyll-a for green leaves in the 6 ppt and 12 ppt treatments were not different from the control treatment in the “Salinity Stress” experiment, nor were there difference between the 6 ppt and 12 ppt treatments in the “Salinity Stress” and the “Stepwise Acclimation” experiments (Table 2; Figure 7). *Vallisneria americana* showed no changes in chlorophyll -a and -b per unit leaf area and maintained maximum electron transport rate at different salinities (0 to 15 ppt natural water salinities) (French and Moore 2003). No difference in concentrations of chlorophylls per unit ash-free dry weight were reported for *V. americana*, *H. verticillata*, *M. spicatum*, and *P. perfoliatus* in salinities (0 to 12 ppt), and nitrogen-based compounds were suggested to be osmoregulators ameliorating increased in cell Na<sup>+</sup> toxicity (Twilley and Barko 1990).

On the contrary, reductions in chlorophyll concentrations, photosystem damage, and decreases in maximum quantum yield and maximum electron transport rate have been observed in *S. natans*, which could not maintain ionic homeostasis (high  $\text{Na}^+/\text{K}^+$  ratio) at 50 mM NaCl and above (Jampeetong and Brix 2009). Substantial reductions in chlorophyll-a and -b and carotenoid, in parallel with high levels of antioxidant defence, were reported for duckweed (*Spirodela polyrhiza*), and aquatic ferns (*Pistia stratiotes* and *Salvinia molesta* L.) when stressed in 200 mM NaCl (Upadhyay and Panda 2005, Chang et al. 2012). In this study, as green leaves in *S. pectinata* gradually turned yellow then black, they became incapable of photosynthesis.

In this study, photosynthesis in relatively younger, fully expanded green leaves of *S. pectinata* seems well protected against salt stress, at least for the period before leaf senescence. Protection of photosystems under salt stresses suggests the maintenance of cytoplasmic  $\text{K}^+$  concentrations and a high  $\text{K}^+:\text{Na}^+$  ratio (James et al. 2006, Garrote-Moreno et al. 2015). As leaf grows, increasing volume could help dilute the in-cell salt concentrations (Richardson and McCree 1985). In fully expanded leaves of *S. pectinata*, maintenance of photosynthesis implies active detoxification of intracellular  $\text{Na}^+$  in new leaves, likely by sequestering  $\text{Na}^+$  in vacuoles and synthesis of organic osmolytes (Rout and Shaw 1998, Garrote-Moreno et al. 2015), such as glutamic acid (unpublished data mentioned in van Wijk, 1989). As leaves age, salt sequestration may finally exceed the vacuole capacity and salt starts to accumulate in the cytoplasm until it reaches a toxic level, causing leaf senescence (Husain et al. 2003, Läuchli and Grattan 2007, Munns and Tester 2008). *S. pectinata* is unlikely equipped with salt exclusion mechanisms to detoxify intracellular  $\text{Na}^+$  because salt exclusion is normally found in species without enhanced leaf senescence, such as in *C. nodosa* (Garrote-Moreno et al. 2015), and in marine cyanobacteria (Hagemann 2011). More likely, the mechanisms for limited salt-tolerance in *S. pectinata* is salt sequestration and, sequentially, leaf shedding to avoid progressively increasing maintenance cost for osmoregulation (Suarez and Medina 2005).

### ***DW: FW Ratios and the Importance of Underground Biomass***

In different aboveground plant tissues, DW:FW ratios were higher for plants at 12 ppt than at 6 ppt. The higher dry matter content probably was due to higher concentrations of organic osmolytes (Parida and Das 2005, Läuchli and Grattan 2007, Munns and Tester 2008, Janda et al. 2016) and higher salt concentrations (Twilley and Barko 1990). For the belowground tissue, however, DW:FW ratios in the end were not different between 6 ppt and 12 ppt salinity treatments in the stepwise acclimation experiment, even though the sediment pore water had the same salinities as the treatments (data not shown). This implies a different ion exchange mechanism in root and rhizome tissues as compared to the more salinity vulnerable tissues such as leaves and stems, and also probably suggests the belowground tissues, an important plant food reserves, could survive at salinities lethal to shoots and provide resilience to salinity damage in *S. pectinata*. In our resilience experiment, exposure to 20 ppt for 3 and 7 days caused irreversible shoot death and it was the underground biomass that generated new shoots after returning to freshwater. The leaf senescence rate in the newly established shoots was not affected by the previous exposure to lethal salinity levels. This suggests in an exposure event to salinity levels of extremities, the underground biomass plays a key role in re-establishing the plants.

### ***Direct Acclimation versus Stepwise Acclimation***

Upon direct salinity exposure, *S. pectinata* showed an increasing leaf senescence rate with higher salinities (Figure 6c). However, in the stepwise acclimation, no difference in leaf senescence rate was found between plants at 6 ppt and at 12 ppt (Figure 5c). Through direct acclimation, the increase in salinity by 12 ppt imposes a stronger osmotic stress and perhaps induces faster accumulation of cytoplasmic ionic concentrations than the increase by 6 ppt, and the detoxification process — such as synthesis of osmoregulatory compounds — possibly could not catch up with the faster increase in cytoplasmic ion concentrations in 12 ppt than in 6 ppt, therefore inducing higher leaf senescence rates. Through stepwise acclimation, a further increase in salinity by 6 ppt did not trigger an increase in leaf senescence compared to the 6 ppt group, instead, it reduced the leaf production rates (Figure 5c). This was likely a diverting of energy to speed up the detoxification process that could handle the increment

in cytoplasmic ion content, resulting in no increased leaf senescence. Physiologically, photosynthetic rates per unit chlorophyll-a and pigments concentration in 12 ppt did not differ from those in 6 ppt through either of the acclimation pathways. This indicates relatively younger, fully expanded green leaves through either of the acclimation pathways are tolerant to 12 ppt. Although both the acclimation pathways generated the same degree of tolerance to 12 ppt salinity, the direct acclimation pathway involved more leaf loss. A previous study of salinity stress on *P. tricarinatus* supports the idea that different acclimation pathways resulted in a same salt tolerant state, but early acclimation to salinity involved much less leaf loss (Warwick and Bailey 1998).

### ***Ecological Implications***

In this study, *S. pectinata* grew and survived in salinities up to 12 ppt, and this range agrees with the previous studies that the plant could grow below 10 ppt and endure salinities up to 16 ppt (van Wijk et al. 1988, Hall et al. 1997, Shili et al. 2007, Borgnis and Boyer 2016). *S. pectinata* did not survive at 20 ppt that could be defined as the threshold salinity level for death. Salinity in Te Waihora (Lake Ellesmere) is between 5 ppt and 10 ppt most of the time that is suitable for growth of *S. pectinata*. Occasional extremely high salinity ( $\geq 20$  ppt) in the lake would cause irreversible shoot death, but after exposure to 20 ppt for 7 days *S. pectinata* is still resilient: new shoots can grow upon returning to freshwater.

Although photosynthesis was not affected by the salinity treatment in relative young, fully expanded green leaves, *S. pectinata* under salinity stress demonstrated reduced leaf production rate with increasing salinities, salt-induced leaf senescence, and reduced leaf length at 12 ppt, all of which contribute to less photosynthetic leaf area. This likely results in less carbon assimilation to sustain growth, particularly in Te Waihora where shoots are fully submerged in the water column with very poor light conditions.

Stepwise salinity increase (0 to 6 to 12 ppt) allowed *S. pectinata* to acclimate with much less leaf senescence compared to an abrupt salinity increase (0 to 12 ppt). This suggests a fully expanded leaf of *S. pectinata* probably could physiologically acclimate (via osmoregulation and  $\text{Na}^+$  detoxification) to a salinity increment by 6 ppt or less, while fail to acclimate to a

salinity increment by 12 ppt. Therefore, gradual salinity increase in Te Waihora unlikely increase leaf senescence rate for *S. pectinata*.

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## Chapter 4: The effects of salinity on photosynthesis in *Stuckenia pectinata* under high and low light conditions

### Abstract

An extensive submerged macrophyte bed (dominated by *Stuckenia pectinata* and *Ruppia megacarpa*) existed around the margins of Te Waihora (Lake Ellesmere), a coastal lagoon on the east coast of the South Island of New Zealand prior to its destruction in a storm event in 1968. Since then submerged macrophytes have never recovered to the prolific, pre-storm state. Rapid light attenuation in the turbid water column and fluctuating salinity are hypothesised as factors limiting the recovery of *S. pectinata*. To understand the dynamics of photosynthesis in *S. pectinata* under combinations of light and salinity stress, a laboratory experiment was conducted. *S. pectinata* plants were first acclimated to three irradiances:  $340 \pm 20 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ,  $110 \pm 10 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ , and  $45 \pm 5 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ . At each irradiance, salinity was increased in a stepwise manner (0, 6, 12 and 18 ppt), with a control treatment remaining in freshwater. Photosynthesis-irradiance relationships, chlorophyll-a and total carotenoids content, and non-photochemical quenching were determined. Leaf photosynthesis per unit fresh weight increased with increasing irradiance, and salinity only constrained leaf photosynthesis per unit fresh weight at 12 ppt and 18 ppt, and then only at the highest irradiance level. Salinity treatment had no effect on photosynthesis at the lower irradiance. Salinity increased chlorophyll-a per leaf fresh weight except at high irradiance. Light-saturated photosynthesis per unit chlorophyll-a was reduced in 12 and 18 ppt under high irradiance, and in 18 ppt under medium irradiance. Coincidentally reduced chlorophyll-a: carotenoid ratios and higher non-photochemical quenching ability was found in the same salinity treatments, suggesting enhanced energy dissipation via heat loss in photosynthetic apparatus. This study indicates that in Te Waihora (Lake Ellesmere), salinity at 12 ppt and above reduces leaf photosynthesis when leaves approach the water surface, while salinity up to 18 ppt has no effects on photosynthesis when leaves are light-limited in lower parts of the water column.

### Key Words:

photosynthesis, turbidity, salinity, chlorophyll-a, non-photochemical quenching

## Introduction

Te Waihora (Lake Ellesmere) is a large, shallow lagoon located on the east coast of South Island of New Zealand. The lake has a highly turbid water column resulting from wind-generated sediment resuspension and eutrophication (Gerbeaux and Ward 1991). The attenuation coefficient in the lake averages around  $9\text{ m}^{-1}$  (Gerbeaux and Ward 1991), and this was within the range of monthly field sampling undertaken in 2016. Such high attenuation rate means 1% of incident irradiance reaches to only 0.5 m water depth. The lake is periodically open to the sea and salinity ranges from 2 to 27 ppt, with an average of 8.4 and standard deviation of 3.8 ppt (2010 to 2016 data from Environment Canterbury).

Historically, an extensive surface-reaching aquatic macrophyte bed occurred in much of the marginal area of the lake, but this was destroyed by a storm event in 1968 and has never recovered to its pre-storm state (Gerbeaux 1993, Jellyman et al. 2009). The primary limiting factors for the macrophyte recovery are hypothesised to be the low light availability in the water column, strong wave action in shallow, better-illuminated waters, and fluctuating salinity (Gerbeaux 1989). *Stuckenia pectinata*, a major species of the historic macrophyte bed, is considered tolerant of turbidity (Gerbeaux and Ward 1991) and of salinity (Jellyman et al. 2009). It is therefore regarded as a key species for any attempt to re-vegetate the lake marginal area.

The previous two research chapters focused on the growth and photosynthesis of *Stuckenia pectinata* in response to one of these factors. Chapter 2 showed that low-light stimulates *S. pectinata* to produce longer stems, leaves and hence overall plant lengths which, in the turbid water column, would allow photosynthetic tissues to reach the water surface sooner to escape low-light stress. Chapter 3 demonstrated that *S. pectinata* produces leaves slower and senesces leaves faster in salinity treatments, and reduces leaf length at high salinities ( $\geq 12$  ppt). This delays the plants reaching the water surface and constrains photosynthetic leaf area in the turbid, low-light water column.

Physiologically, adverse effects on various parts of photosynthesis have been reported for other aquatic or marine algae or plants. Salt stress reduces content of photosynthetic pigments and enhances antioxidant defence in *Spirodela polyrrhiza*, *Pistia stratiotes*, and *Salvinia molesta* L. (Jampeetong and Brix 2009, Chang et al. 2012). Dehydration due to high

salinity in tissue of algae *Porphyra perforate* limits electron flow on the reducing site of photosystem I and the water side of photosystem II, and slows down transfer of excitation energy between photosynthetic pigments (Satoh et al. 1983). Salinity reduces oxygen production rates in marine green algae *Dunaliella tertiolecta* temporarily before successful osmoregulation (Gilmour et al. 1982) and inhibits electron transport at the reducing site of photosystem II in *Ulva lactuca* (Xia et al. 2004). Incapability to maintain a cytoplasm ionic balance under salinity stress causes dysfunction of photosynthesis in *S. natans* (Jampeetong and Brix 2009).

Salinity treatment at 6 ppt and 12 ppt illuminated with  $100 \mu\text{mol m}^{-2} \text{s}^{-1}$  PAR, however, did not exert adverse effects on photosynthetic pigments nor on photosynthetic rate per unit chlorophyll-a (Chapter 3). No adverse effects of salinity on photosynthesis is due to that only leaves before the start of salt induced senescence were investigated, or perhaps due to salinity level or illumination was not high enough. In Te Waihora, leaves of *S. pectinata*, positioned at different water depth, experience a gradient of irradiance levels from full sunlight at the water surface attenuating to semi-darkness in the water column, and in salinities ranging from 2 ppt to a lethal level above 20 ppt.

Satoh et al. (1983) discussed that limited electron flow through photosystems was a measure of protection against photosystem damage. If salinity affects any procedures of electron flows down stream of PS II, the photosynthetic apparatus will respond as if in acclimation to high light stress (Anderson et al. 1995, Huner et al. 2012). Then we hypothesized that potential interaction of high irradiance and salinity on photosynthesis is to reduce photosynthetic rates and to enhance NPQ (Huner et al. 2012). If salinity affects PSII itself, we would likely detect reductions in the intrinsic quantum yield.

This study set out to investigate how increasing salinity stress affects photosynthesis of relative young and fully expanded green leaves in *S. pectinata* under a gradient of light intensities, which represent different optic depth along the turbid column in Te Waihora. The average daily global radiation for the lake during the growing season (November to March) is estimated to be  $19.3 \text{ MJ m}^{-2} \text{ d}^{-1}$  (NIWA Climate Data, 1981-2010), that is approximately  $470 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$  after conversion to photosynthetically active radiation (PAR). The average attenuation coefficient is  $9 \text{ m}^{-1}$  for down-welling radiation

(Hawes and Ward 1996). This predicts  $100 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$  at 0.2 m deep and  $50 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$  at 0.4 m deep.

## **Materials and Methodology**

### ***Experiment Design***

The effects of salinity on photosynthesis in *Stuckenia pectinata* were investigated at three levels of photosynthetically active radiance (PAR):  $340 \pm 20 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ,  $110 \pm 10 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ , and  $45 \pm 5 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ . Hereafter these are referred to as high light (HL), medium light (ML), and low light (LL), respectively. All plants were acclimating to the assigned irradiance levels in freshwater for 20 days before the implementation of salinity treatments. After acclimation to irradiance, two salinity treatments were applied. One was freshwater (control) treatment kept at 0 ppt, the other was salinity treatment that involved a stepwise increase in medium salt content such that plants experienced 6, 12 and 18 ppt, for 2 weeks at each salinity prior to each increase. At HL and ML, there were 8 plant replicates per treatment assigned randomly and evenly among four treatment replicates; at LL, there were 5 plant replicates distributed among three treatment replicates: two containing 2 plant replicates and one containing 1 plant replicate. Each treatment replicate comprised 4 L of plant culture medium (tap water containing 0.015 mg /L dissolved reactive phosphate; 1.5 mg /L nitrate-nitrogen; 61 mg/L bicarbonate) in a 22 cm deep white plastic container. At each irradiance level, plant replicates were randomised among treatment replicates weekly. For salinity treatments the medium comprised tap water and sea salt (Instant Ocean®, USA) adjusted to the targeted salinity level, measured with an YSI ECO Sense EC300A conductivity meter (Xylem®, USA). At the end of the second week at each salinity level, light induction curves, photosynthesis-irradiance curves, leaf dry to fresh weight ratios, and chlorophyll-a and total carotenoids concentrations were analysed using young, fully expanded leaves. At HL and ML six replicates were run but limitations in the amount of material growing at LL restricted replication to N=5. Samplings at the 6 ppt, 12 ppt, 18 ppt are referred as the first, second and third sampling respectively.

### **Plant Material**

The *Stuckenia pectinata* used for HL and ML were derived from a greenhouse population cultured in freshwater (Volume: 3.2 m<sup>3</sup>, Depth: 0.6 m). Each replicate plant was a healthy shoot of 30 to 50 cm height, cut at the sediment-water interface, and with its basal leaf removed to expose an internode. The shoot was then re-planted with the internode buried in sediment in plastic beakers (4.5 cm in diameter and 5.5 cm tall). The sediment was a mixture of fine sand and clay with a volume ratio of 2:1; the fine sand was collected from a beach near Te Waihora (Lake Ellesmere) and the clay was sieved sediment from the lake. A thin layer of coarse sand was sprinkled over each. The replanted shoots were kept in the greenhouse, and over a two-month period new rhizomes developed from the internode and sent up new shoots. The old shoots were cut and new shoots were kept and transferred to a growth room. The chosen plants at the beginning of this experiment had 2 to 4 shoots and 9 to 20 leaves. The plant material for the LL treatment were germinated tubers in the sediment in beakers as described above that had been raised in the growth room under 50  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  LED light for 2 months. At the beginning of the experiment, they had 1 to 3 shoots and 6 to 21 leaves.

### **Growth Conditions**

The growth room was maintained at a constant temperature of 20 °C. Two 100 W LED lights (colour temperature 4000 K) were mounted above the plastic containers providing PAR (photosynthetically active radiation) with a day-night cycle of 12 h:12 h. PAR was measured with a Li-Cor Li192 Quantum Sensor connected to a LI1500 data logger (Li-Cor, Inc., US) at the water surface where leaves of *S. pectinata* were floating. Different irradiances were achieved by varying the distance between the surface of the growth medium and the LED lights. Growth medium was bubbled with ambient air continuously at a rate that did not disturb the positions of leaves in the water column. pH was monitored twice a week and any medium measured pH above 8.5 was replaced with tap water and adjusted to the targeted salinity. Tap water was added every two days to compensate evaporation loss and the culture medium was replaced for all plants every two weeks when salinity was increased for the salinity treatments.



### ***Chlorophyll-a and Total Carotenoid Concentration***

For each plant replicate, a young and fully expanded leaf was excised and weighed for fresh weight. Within an hour of collection, each was ground with a mortar and a pestle in 5 mL 90% acetone for 30 seconds, before the extracts were transferred into a 15 mL plastic vial. This step was repeated twice to ensure a complete extraction. After storing at 6°C for 24 hours, the extracts were centrifuged at 3000 r.p.m for 15 minutes. The absorbance of the supernatant was then measured at 470 nm, 647nm, 664nm, and 750nm (Spectrophotometer DR3900, HACH®, USA) in a 1 cm glass cuvette. Chlorophyll-a concentration was calculated according to Porra (2002) and total carotenoid concentration was estimated according to Wellburn (1994). Chlorophyll-a to total carotenoid ratio was calculated.

### ***Photosynthesis – Irradiance (PI) Curves***

PI curves were carried out at each acclimated light treatment (HL, ML, LL), each salinity (0, 6, 12, 18 ppt) with replications. Leaves for PI curve analysis were collected before the start of the light cycle, and therefore were fully dark-adapted. All measurements were made on two consecutive days, one day for all the freshwater treatments and the other day for all the salinity treatments of the three irradiance levels.

For each leaf collected, oxygen production rates were measured in the dark and under a series of increasing PARs. Darkness was created by wrapping samples with aluminium foil. Irradiance was provided by three, radially arranged 100 W LED floodlights (colour temperature 4000 K), and placed 50 cm above a temperature-controlled water bath. The irradiance was manipulated using layers of white translucent cloth and two polycarbonate boards placed 30 cm below the LED lights. This arrangement increased light scattering and provided a homogeneous light field for plant incubations in the water bath. A LiCor Li-192 quantum sensor was used to measure PAR. A gradient of PARs for PI curve analysis was 0, 21, 46, 66, 89, 129, 192, 242, and 317  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ .

Each element in the water bath (the water bath container, the water pump, the heating unit, and the rack) was black to prevent reflections. A circular area, 23 cm in diameter at the

surface of the water bath, was illuminated almost homogenously (Table 1) and could accommodate up to 22 incubation vials on a rack 1 cm below the water surface. The water bath was maintained between 20.4 and 20.8 °C and circulated using a submersible pump (23 w, 1001, EHEIM, GmbH).

Incubations were of a single leaf sealed in a 12.2 mL glass vial, filled with air-saturated tap water with the required salinity, enriched with 10 mmol/L bicarbonate to prevent carbon depletion affecting the photosynthetic rate. Three blank glass vials were incubated each time as controls. At the end of each incubation, the glass vials were well mixed (two 5 mm diameter glass beads had been placed in each vial to assist in-vial water mixing), and oxygen concentrations were estimated using a PreSens Oxygen Microsensor connected to a Microx 4 control unit (PreSens GmbH, Germany). Initial incubations were in darkness, after which the leaves were incubated incrementally over a gradient of irradiance, with the incubation time determined by the intensities of actinic light levels (Table 1).

Table.1 Light treatments and incubation times used for determination of photosynthesis-irradiance relationships. (For each PAR treatment, incubation area PAR was presented with mode and range)

Light Level	1	2	3	4	5	6	7	8
Mode ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )	21	46	66	89	129	192	242	317
Range ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )	2.6	3.6	1.8	1.8	3.0	3.0	9.0	14.0
Incubation time (s)	3600	3600	2700	2700	1800	1800	1800	1800

After all incubations were complete, leaves were measured for fresh weight (FW), then dried at 80 °C for 24 hours, cooled, and dry weight (DW) determined. For fresh weight measurement, a leaf sample was taken out of the incubation glass vial, each side of the leaf was dried on tissue paper for 5 seconds, and was immediately and gently located on a balance. The fresh weight of a leaf sample decreased with time (by 0.0001 g every 2-3 seconds) due to evaporation loss, and the weight of the first 2 seconds on balance was taken as leaf fresh weight. The leaf sample after FW measurement could be rehydrated in the glass vial and used to repeat the FW measurement to yield result with difference less than 0.0002 g (FW of leaf samples range from 0.01 to 0.03 g). The DW: FW ratio was calculated for each leaf. Oxygen production was calculated as the difference between

oxygen concentrations relative to the average of the empty control glass vials and, for reasons discussed below, normalised to incubation time and leaf fresh weight ( $\mu\text{g O}_2 \text{ g}^{-1} \text{ FW s}^{-1}$ ) and chlorophyll-a ( $\mu\text{g O}_2 \text{ mg}^{-1} \text{ Chl-a s}^{-1}$ ).

### ***Light-Induction Curves***

The induction kinetics of chlorophyll-a fluorescence was investigated using new fully expanded leaves 3 hours before the start of the light cycle. The investigation was carried out on two consecutive days, with the first day for control treatments and the second day for the salinity treatments. A Junior-PAM Fluorometer (Walz GmbH, Germany), controlled by the WinControl3 System Control and Data Acquisition Programme (Walz GmbH, Germany) was employed. The fluorometer delivered measuring beams, saturating pulses and actinic light, all of which were delivered from a blue LED light source centred on 445 nm wavelength, via a 1.5 mm diameter optic fibre. A fully dark-adapted leaf was gently held just above the water surface, whereupon the optic fibre was positioned so that the tip of the fibre was perpendicular to the leaf surface and almost touching the leaf. The PAM fluorometer provided a weak measuring light modulated at a frequency of 10 Hz. When the baseline fluorescence signal ( $F_0$ ) became stable the “Light Induction Curve” programme in the WinControl3 was initiated. A 1.2 s,  $4660 \text{ m}^{-2} \text{ s}^{-1}$  saturating pulse was applied and the dark-adapted maximum fluorescence ( $F_m$ ) was obtained. 40 s after the first saturating pulse, actinic light ( $150 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ) was switched on and simultaneously saturating pulse analysis was applied fifteen times at 25-second intervals. Fluorescence yield correspondent to the modulated measuring light was recorded through the time.

From the recorded fluorescence data, the following fluorescence yield parameters registered by the programme were extracted. From the initial saturating pulse analysis,  $F_0$  and  $F_m$  were investigated --  $F_0$  is the basic fluorescence yield when the plastoquinone pool that serves to transfer electrons from PSII to PSI is fully oxidised in dark-adapted leaves, and  $F_m$  is the maximum fluorescence yield under fully reduced plastoquinone conditions in dark-adapted leaves where it was assumed that there was no non-photochemical quenching. After the onset of actinic light,  $F'$  – the fluorescence yield immediately before the saturating pulses, when the plastoquinone pool was reduced under ambient actinic light, and  $F_m'$  - the

maximum fluorescence yield when the plastoquinone pool was reduced under the actinic light, and when non-photochemical quenching may have been initiated in response to elevated pH gradients within the chloroplasts (Königer et al. 1995). From those parameters, maximum quantum yield and Stern-Volmer non-photochemical quenching (NPQ) were calculated as:

$$\text{Maximum Quantum Yield: } (F_m - F_o) / F_m$$

$$\text{Stern-Volmer NPQ: } (F_m - F_m') / F_m'$$

### ***Data Analysis***

For the DW: FW ratios, photosynthetic rate per unit FW, pigment concentrations and ratios, NPQ coefficient and maximum quantum yield, differences within control plants and within salinity plants were investigated using repeated measures ANOVA. Differences within the control plants were partitioned into differences due to irradiance levels, differences due to effects from unexpected factors (named “control” factor) that may or may not result in changes in the control group among the three sampling times, and differences due to irradiance and control interactions. Differences within the salinity plants were partitioned into differences resulting from irradiance level, salinity level, and their interactions. For the repeated measures ANOVA, the normality of the residual of the model fitting was confirmed with a Shapiro-Wilk test (Shapiro and Wilk 1965) and data were transformed if necessary to assure normality of the residuals. A Tukey HSD test using least square means was used to identify significant effects if a significant difference was found. Subsequently, the effect of each salinity level at each irradiance level was analysed by comparison to the correspondent freshwater treatment using a t-test, assuming non-equal variance.

For the photosynthetic rates per chlorophyll-a versus irradiance data, photosynthetic rates at each actinic light level in the control treatments were compared to determine the effects of irradiance with one-way ANOVA across the three irradiance levels, and subsequently using the Tukey HSD test. The difference between salinity treatment and the corresponding control at each actinic light level was compared with a t-test assuming non-equal variance. Hyperbolic Tangent curves (Equation 1) (Jassby and Platt 1976) were fitted to

photosynthesis-irradiance relationship for each treatment for presentation purposes and to obtain representative values of key metrics describing the relationship, using a non-linear least square algorithm.

$$P = P_{\max} * \tanh (\alpha * I / P_{\max}) + R \quad (\text{Equation 1})$$

Where:

P: net photosynthetic rate, as a dependent variable

I: photosynthetic active radiation, as an independent variable

$\alpha$ : apparent quantum yield, slope of the curve at low irradiance before the onset of maximum photosynthesis

$P_{\max}$ : light-saturated (maximum gross) photosynthetic rate

R: dark respiration rate

Saturating irradiance ( $E_k$ ) for photosynthesis was estimated by  $P_{\max} / \alpha$  for each curve. All analyses were carried out using JMP Software (Version 9, SAS®, USA).

## Results

### ***Dry weight to fresh weight ratios***

Dry weight accounted for  $8.7\% \pm 1.5\%$  (median plus/minus one quartile range) of the leaf fresh weight in all leaf samples. For control plants, DW: FW ratio increased with increasing irradiance levels (repeated measures ANOVA,  $p < 0.001$  and Tukey HSD), and was higher at the third sampling than the first and second samplings (repeated measures ANOVA,  $p = 0.001$  and Tukey HSD) (Figure 1). There was no effect of interactions between irradiance and control factor on the DW: FW ratio.

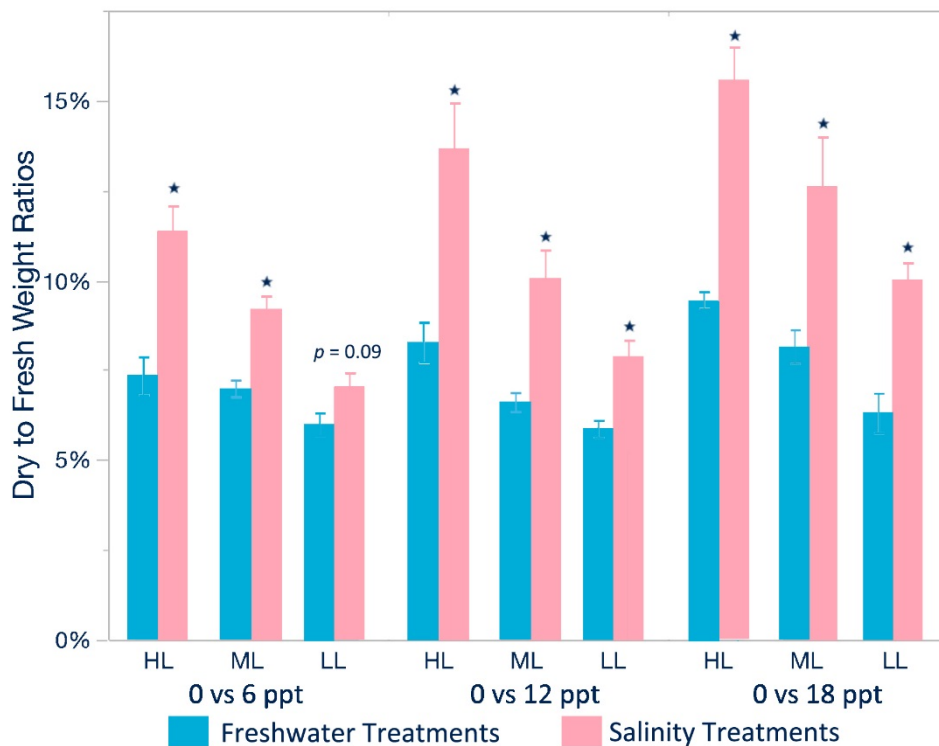


Figure 1. Leaf dry weight (DW) to fresh weight (FW) ratios (Error bar is standard error of the mean; a significant difference between the salinity treatment and the corresponding control treatment was indicated by a star).

For salinity plants, DW: FW ratio of the salinity groups increased with increasing irradiance (repeated measures ANOVA,  $p < 0.001$  and Tukey HSD), was higher at the 18 ppt than at the 6 and 12 ppt treatments (repeated measures ANOVA,  $p = 0.0003$  and Tukey HSD), and was not influenced by irradiance and salinity interactions (repeated measures ANOVA,  $p = 0.93$ ).

Salinity treatments increased the DW: FW ratio at all irradiance levels and for all salinity level comparisons (Figure 1). In comparison to the corresponding controls, salinity treatment at 6, 12, and 18 ppt increased the DW: FW ratios by 54%, 65%, and 64% respectively for HL plants, by 32%, 53%, and 55% respectively for ML plants, and by 17%, 35%, and 59% respectively for LL plants. The increase in DW: FW ratio was more rapid with increasing irradiance upon exposure to the first salinity level at 6 ppt.

***Normalising photosynthesis, respiration, and pigment contents***

Changes in the DW: FW ratio complicated the choice of how to normalise other measured parameters such as leaf photosynthetic rates and pigment concentrations. Leaf DW: FW ratio varied with both irradiance and salinity, and in salinity treatments DW may contain accumulated and sequestered salt, and hence bias DW-specific parameters. Therefore, key parameters were normalised to FW. As  $91.3 \pm 1.5\%$  (median plus/minus one quartile range) of fresh weight was water for all sampled leaves, standardising to leaf fresh weight was similar to standardising to unit leaf volume. Leaf width was correlated to neither irradiance levels (Chapter 2) nor salinity levels (Chapter 3) and leaf thickness (0.5 - 1 mm) was less than 1% of leaf length (100 -200 mm), therefore leaf volume is correlated with leaf length that indicates the photosynthetic leaf area. Normalising photosynthesis and pigments content to FW, therefore, approximates to photosynthesis and pigments per unit photosynthetic leaf area.

***Photosynthesis at the treatment irradiance and dark respiration***

For the HL ( $350 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) plants, PI curves showed that leaf photosynthesis was light saturated and the photosynthetic rates at the last two actinic light levels were pooled together to estimate photosynthetic rates at growth irradiance (Figure 2). For the ML ( $110 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) plants, leaf photosynthesis was not fully light saturated or just light saturated at growth irradiance (Figure 2) and photosynthesis measured at actinic light  $129 \mu\text{mol m}^{-2} \text{s}^{-1}$  was used as photosynthesis at growth irradiance. At the low irradiance level ( $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ ), photosynthesis under culture conditions was estimated by photosynthetic measurement at  $46 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Photosynthesis measurements representing photosynthesis at growth irradiance are indicated in Figure 2.

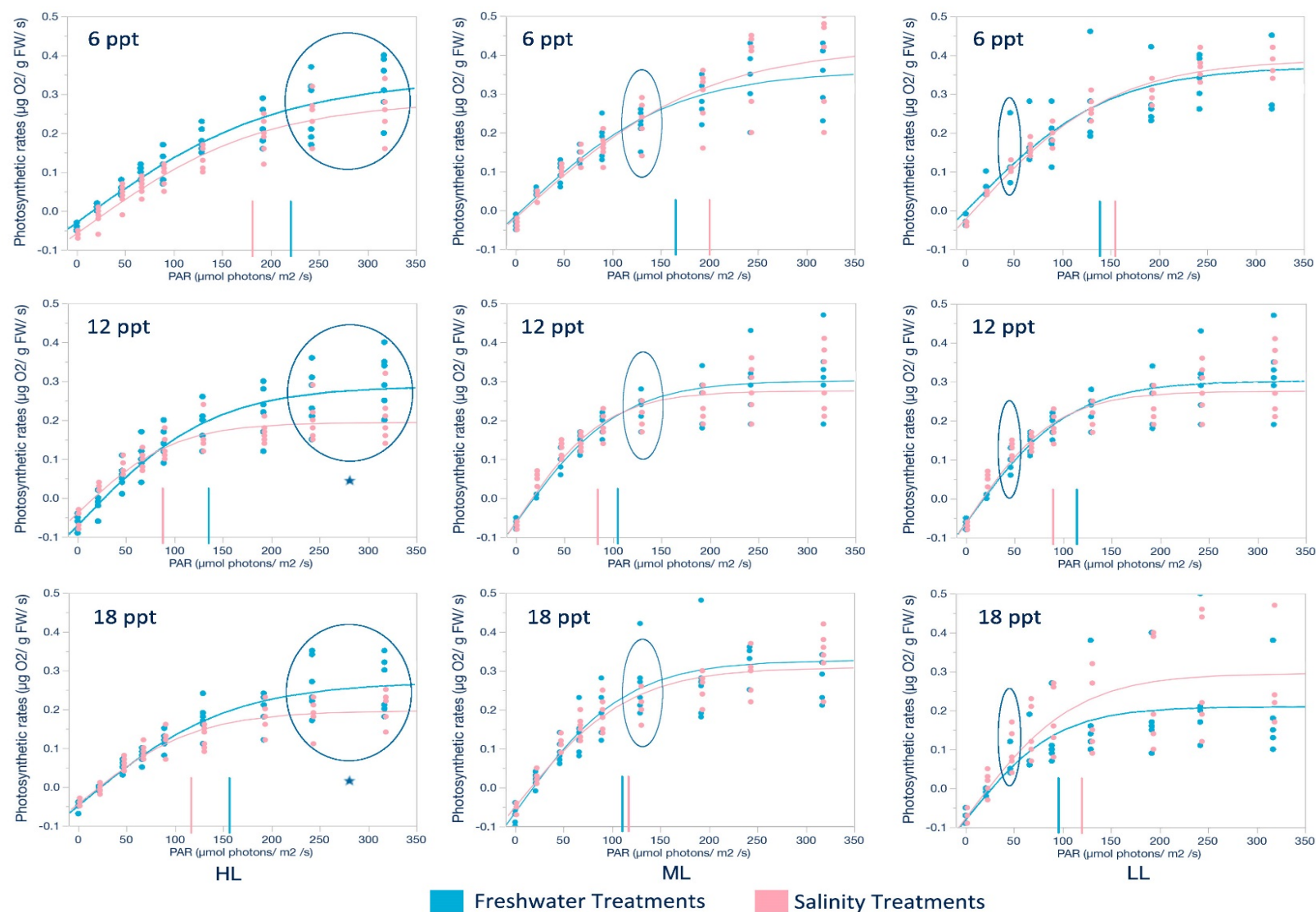


Figure 2. Photosynthesis-irradiance curves standardised to unit leaf fresh weight (bars perpendicular to the x-axis are estimated  $E_k$ , circular areas indicate photosynthetic rates at the growth irradiance, significant difference between the control and the salinity treatment are indicated by stars)



For the control plants, photosynthetic rates at culture irradiance increased with increasing irradiance levels (two-way ANOVA,  $p < 0.001$  and Tukey HSD test). Neither control factor (two-way ANOVA,  $p = 0.68$ ) nor irradiance and control factor interaction (two-way ANOVA,  $p = 0.33$ ) further affected these rates. For the salinity treatment plants, photosynthesis was higher at the HL and the ML than the LL, but did not differ between the HL and the ML (two-way ANOVA,  $p < 0.001$  and Tukey HSD). Salinity (two-way ANOVA,  $p = 0.09$ ), and salinity and irradiance interaction (two-way ANOVA,  $p = 0.45$ ) had no significant effect on leaf photosynthesis. At HL, leaf photosynthesis was constrained by salinity treatments at 12 ppt (t-test,  $p = 0.009$ ) and at 18 ppt (t-test,  $p = 0.01$ ). Salinity treatments had no effects on leaf photosynthesis at the other two irradiance levels.

Dark respiration of the first sampling time was not used in this study because they were underestimated due to light leakage during the dark incubation. Thereafter, for the control plants, leaf dark respiration was not affected by growth irradiance (two-way ANOVA,  $p = 0.32$ ), control factor (two-way ANOVA,  $p = 0.90$ ), or their interaction (two-way ANOVA,  $p = 0.12$ ). For the salinity plants, dark respiration was significantly lower at HL than at ML and LL (two-way ANOVA,  $p = 0.002$ ), and was not affected by salinity (two-way ANOVA,  $p = 0.28$ ) and irradiance salinity interaction (two-way ANOVA,  $p = 0.69$ ). Direct comparisons of salinity treatments vs controls showed no effect on dark respiration (Figure 2).

### ***Leaf Chlorophyll-a concentrations***

For control plants, the chlorophyll-a concentration per unit fresh weight (Chl-a /FW) decreased with increasing irradiance (repeated measures ANOVA,  $p < 0.001$  and Tukey HSD), and was lower at all growth irradiances at the first sampling than the second and third (repeated measures ANOVA,  $p = 0.003$  and Tukey HSD) (Figure 3). There was no interaction between irradiance and control factor on the leaf Chl-a/FW (repeated measures ANOVA,  $p = 0.13$ ). For salinity plants, the Chl-a/FW also decreased with increasing irradiance (repeated measures ANOVA,  $p < 0.001$  and Tukey HSD), was lower at the 6 ppt than the 12 ppt and 18 ppt treatments (repeated measures ANOVA,  $p = 0.0002$  and Tukey HSD), and was affected by irradiance and salinity interaction (repeated measure ANOVA,  $p = 0.04$ ). Chlorophyll-a content did not differ among salinity treatments for the HL plants, while it was higher at 12 and 18 ppt compared to at 6 ppt treatment for the ML plants, and was higher at 18 ppt compared to at 6

and 12 ppt for the LL plants. Salinity treatments increased leaf Chl-a/FW at the ML and LL. At HL, only 12 ppt treatment increased marginally leaf chlorophyll-a concentrations.

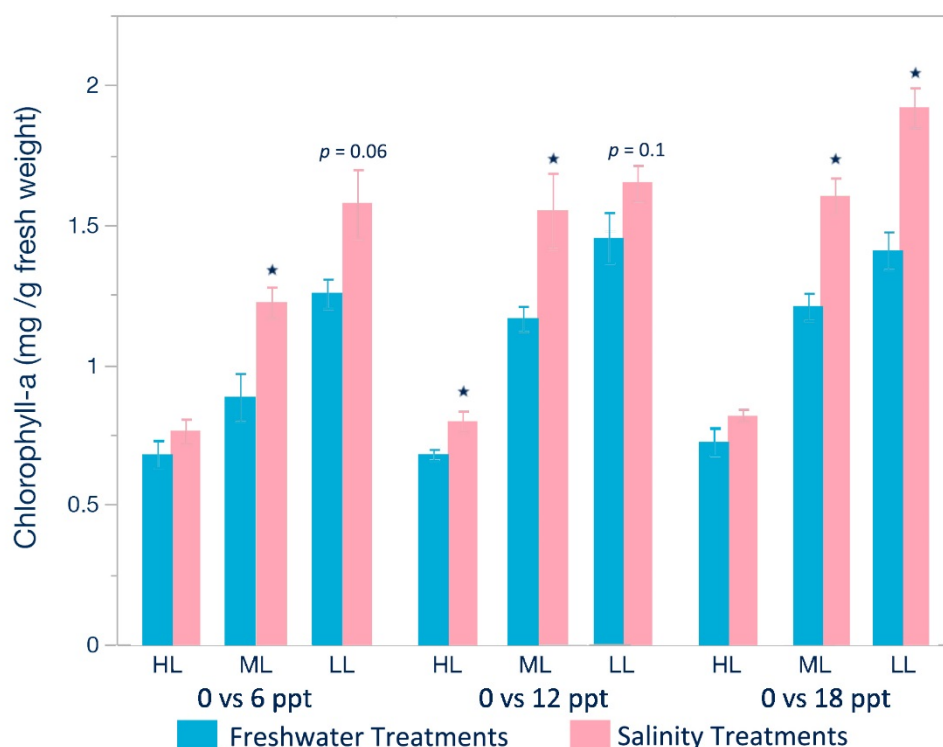


Figure 3. Chlorophyll-a concentrations per unit fresh weight (Error bar is standard error of the mean; significant difference between the salinity treatment and the corresponding control treatment is indicated by a star)

### ***Photosynthesis per unit chlorophyll-a***

Photosynthesis was also normalised to chlorophyll-a concentrations ( $PS_{Chl-a}$ ). For control plants,  $PS_{Chl-a}$  differed among different growth irradiances, and these differences evolved over time. For the first sampling, light-saturated  $PS_{Chl-a}$  was higher for the HL plants than the LL plants (Table 2 and Figure 4). At the second sampling, HL plants showed higher light-saturated photosynthesis and higher light-limited photosynthesis than the ML and LL plants, whereas the ML and LL plants were similar (Table 2 and Figure 4). At the third sampling,  $PI_{Chl-a}$  curves for the HL plants were similar to that for the ML plants, both of which had higher light-saturated photosynthesis and light-limited photosynthesis than the LL plants (Table 2 and Figure 4).

Table 2. Significant differences in photosynthetic rates normalised to chlorophyll-a, at a range of actinic irradiance, among the three irradiance levels (Differences among groups at each actinic light level are indicated by different letters)

"0 vs. 6 ppt"	Actinic level	0	21	46	66	89	129	192	242	317
	High Irradiance	a	a	a	a	a	a	a	a	a
	Medium Irradiance	a	a	a	a	a	a	ab	ab	ab
	Low Irradiance	a	a	a	a	a	a	b	b	b

"0 vs. 12 ppt"	Actinic level	0	21	46	66	89	129	192	242	317
	High Irradiance	a	a	a	a	a	a	a	a	a
	Medium Irradiance	a	a	b	b	b	b	b	b	b
	Low Irradiance	a	a	b	b	c	b	b	b	b

"0 vs. 18 ppt"	Actinic level	0	21	46	66	89	129	192	242	317
	High Irradiance	a	a	a	a	a	a	a	a	a
	Medium Irradiance	a	ab	a	b	a	a	a	a	b
	Low Irradiance	a	b	b	b	b	b	b	b	c

The effects of salinity on  $PS_{Chl-a}$  were reductions in light-saturated photosynthetic rate in 12 and 18 ppt treatments at HL, and in 18 ppt treatment at ML (Figure 4). At HL, salinity treatments (12 and 18 ppt) even started to adversely affect photosynthesis before the onset of light saturation. At the LL, salinity treatments had no effects on  $PS_{Chl-a}$  (Figure 4).

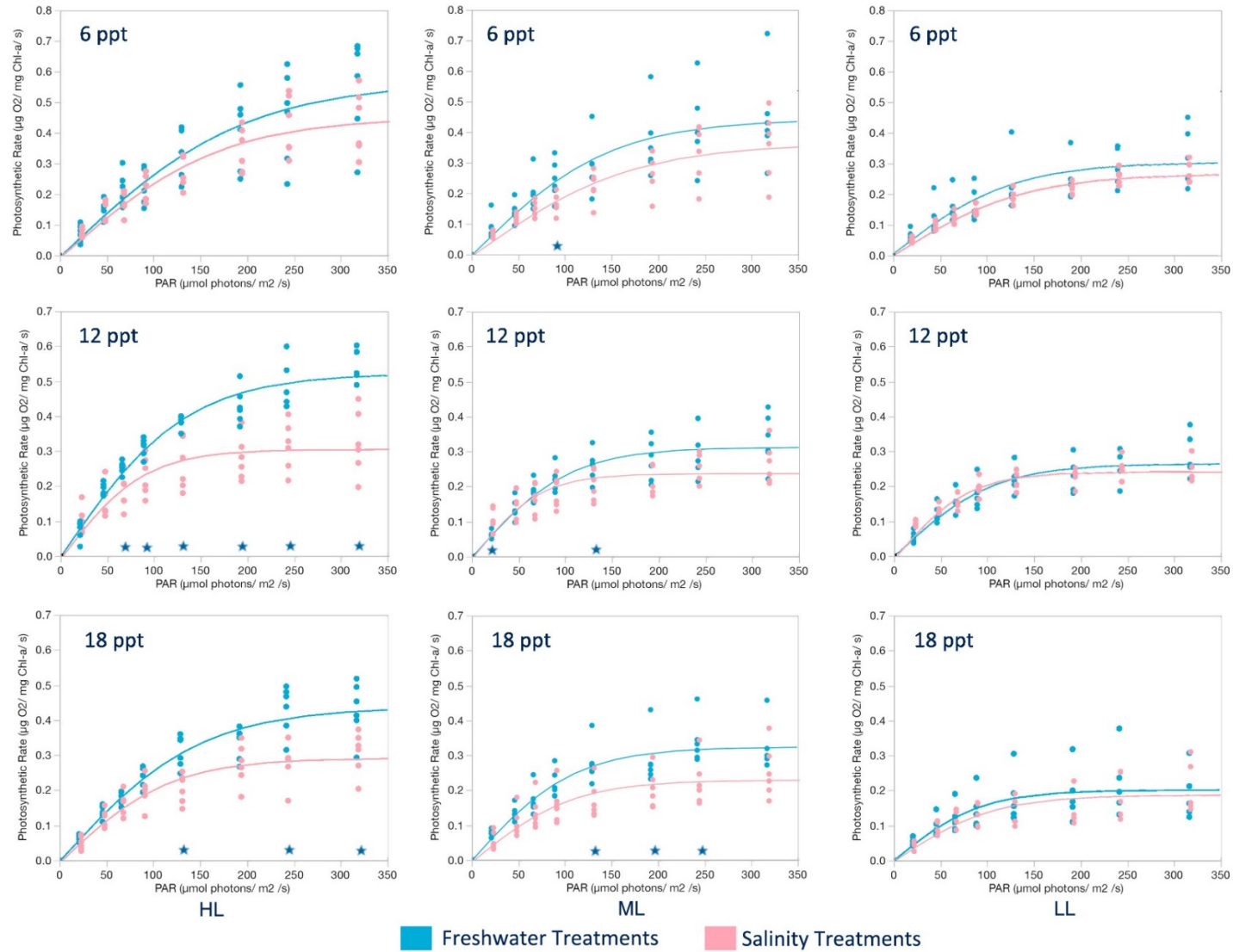


Figure 4. Photosynthesis-irradiance curves standardised to unit chlorophyll-a (significant difference between the control and the salinity treatment at each actinic light level were indicated by stars at the bottom of each graph)

### ***Carotenoid concentration, Chl-a/ Car Ratio, and Non-Photochemical Quenching***

For control plants, total carotenoids concentration per FW was consistently higher at HL than at ML and LL (repeated measures ANOVA,  $p < 0.001$  and Tukey HSD), and was higher at the third sampling than the first (repeated measure ANOVA,  $p = 0.004$  and Tukey HSD). Irradiance and control factor had no interaction effect (repeated measures ANOVA,  $p = 0.052$ ). For salinity plants, total carotenoids concentration also increased with increasing irradiance (repeated measures ANOVA,  $p < 0.0001$  and Tukey HSD), was higher in 18 ppt treatment than 6 and 12 ppt treatments (repeated measures ANOVA,  $p = 0.0003$  and Tukey HSD), and was not affected by irradiance and salinity interaction (repeated measures ANOVA,  $p = 0.91$ ). Compared to control plants, all salinity treatments increased total carotenoids concentration at all irradiance levels (Figure 5).

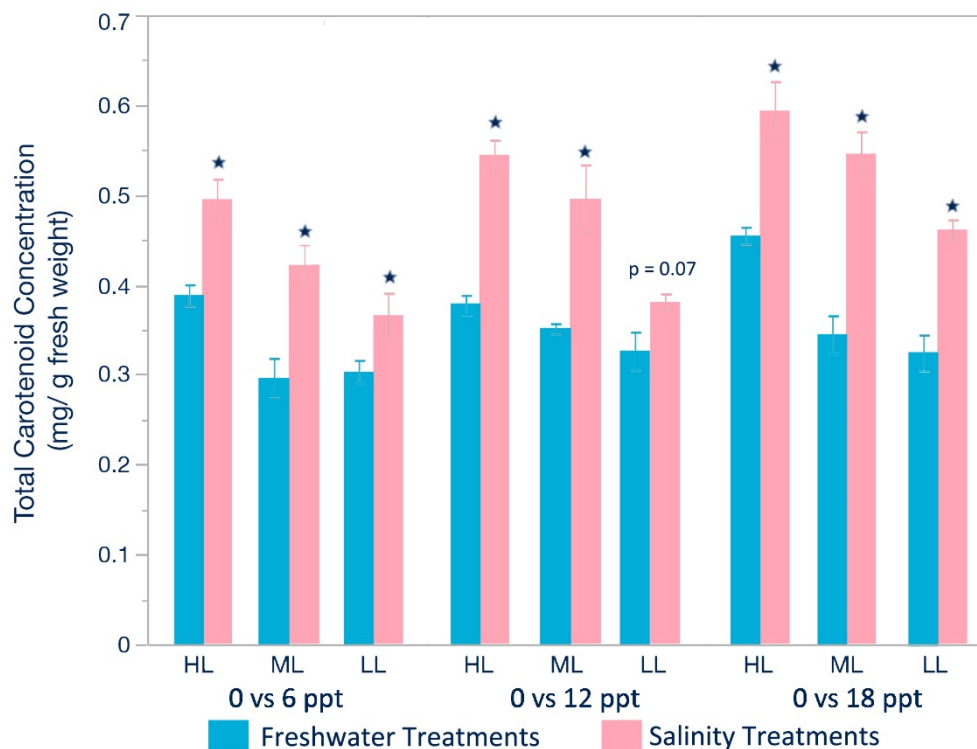


Figure 5. Total carotenoid concentrations (Error bar is standard error of the mean; significant difference between the salinity treatment and the corresponding control treatment was indicated by a star)

In control plants, Chl-a: Car ratio decreased with increasing irradiance (repeated measures ANOVA,  $p < 0.001$  and Tukey HSD), and was not affected by control factor (repeated measures ANOVA,  $p = 0.08$ ) or by irradiance and control factor interaction (repeated measure ANOVA,  $p = 0.08$ ). For salinity plants, Chl-a: Car ratio decreased with increasing irradiance (repeated measures ANOVA,  $p < 0.001$  and Tukey HSD), was not affected by salinity (repeated measure ANOVA,  $p = 0.42$ ) or by irradiance and salinity interaction (repeated measures ANOVA,  $p = 0.77$ ). At HL, 12 ppt and 18 ppt treatments decreased Chl-a: Car by 19% and 12%, respectively. At ML, 18 ppt treatment decreased the ratio by 15%. These differences are not statistically significant but considered biologically significant (Figure 6). At LL, salinity treatment did not alter Chl-a: Car ratio.

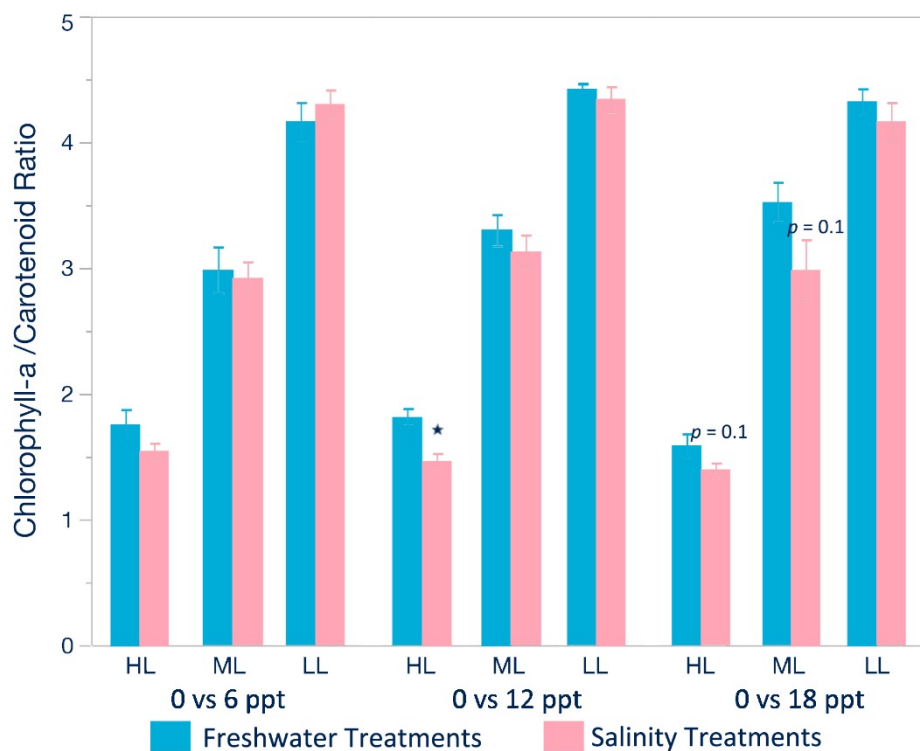


Figure 6. Chlorophyll-a to total carotenoids ratio (Error bar is standard error of the mean; significant difference between the salinity treatment and the corresponding control treatment was indicated by a star)

In control plants, NPQ at the end of the induction period was lower when grown under LL than at ML and HL (repeated measures ANOVA,  $p = 0.002$  and Turkey HSD). NPQ was not affected by control factor (repeated measures ANOVA,  $p = 0.051$ ). Control factors and irradiance interaction affected NPQ (repeated measures ANOVA,  $p < 0.001$ ): NPQ for the HL plants was higher at the third sampling than at the second sampling, NPQ for the ML plants was lower at the second sampling compared to the first, and NPQ for the LL plants did not differ among the three samplings (Figure 7).

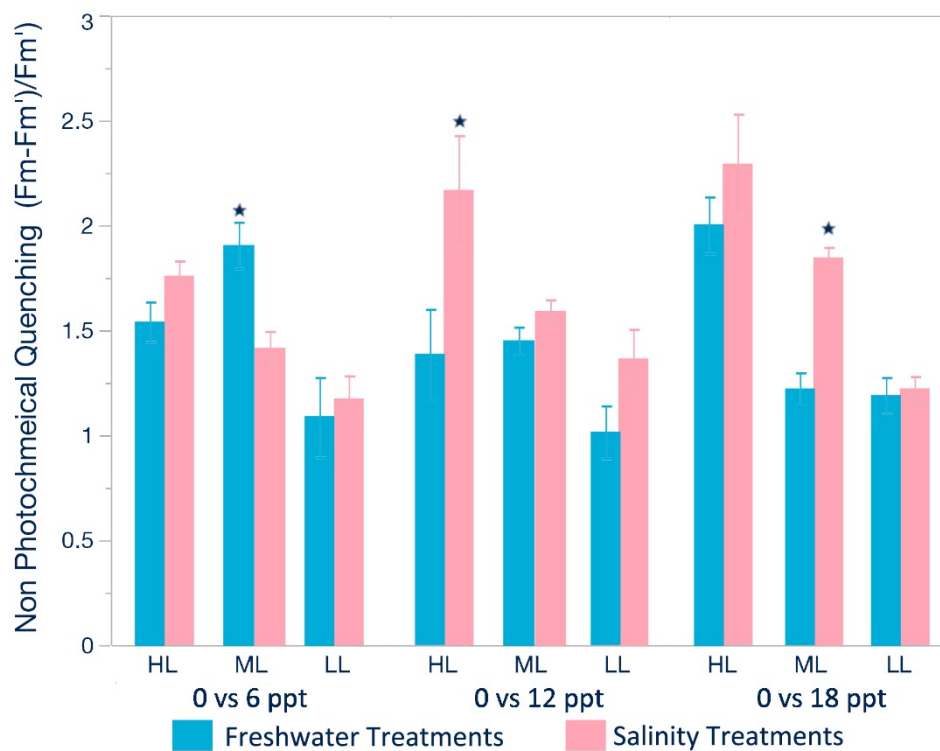


Figure 7. Stern-Volmer non-photochemical quenching coefficient (Error bar is standard error of the mean; significant difference between the salinity treatment and the corresponding control treatment was indicated by a star)

For the salinity plants, NPQ increased with increasing irradiance (repeated measures ANOVA,  $p < 0.001$  and Tukey HSD) and increased with salinity levels (repeated measures ANOVA,  $p = 0.03$  and Tukey HSD), and was not affected by irradiance and salinity interaction (repeated measure ANOVA,  $p = 0.49$ ). Salinity enhanced NPQ in 12 ppt at HL and in 18 ppt and ML (Figure 7).

### **Maximum quantum yield**

Maximum quantum yields were close to 0.8 across all treatments. For the control plants, irradiance (repeated measures ANOVA,  $p = 0.051$ ) and control factor (repeated measures ANOVA,  $p = 0.45$ ) had no effect on maximum quantum yield, nor did their interaction (repeated measures ANOVA,  $p = 0.11$ ). Salinity treatments affected maximum quantum yield in a few treatments (Table 3), but the difference was marginally therefore considered not biologically important, although statistically significant.

Table 3. Maximum Quantum Yield after dark adaptation for 10 hours (mean  $\pm$  standard deviation, the significant difference between the saline and freshwater treatments were indicated by different letters)

	High Irradiance		Medium Irradiance		Low Irradiance	
	Control	Saline	Control	Saline	Control	Saline
6 ppt	0.80 $\pm$ 0.01	0.81 $\pm$ 0.01	0.81 $\pm$ 0.01 <sup>a</sup>	0.83 $\pm$ 0.01 <sup>b</sup>	0.79 $\pm$ 0.03	0.82 $\pm$ 0.01
12 ppt	0.79 $\pm$ 0.01	0.79 $\pm$ 0.02	0.81 $\pm$ 0.01	0.82 $\pm$ 0.01	0.80 $\pm$ 0.02	0.82 $\pm$ 0.01
18 ppt	0.78 $\pm$ 0.02	0.78 $\pm$ 0.01	0.80 $\pm$ 0.02 <sup>a</sup>	0.83 $\pm$ 0.02 <sup>b</sup>	0.81 $\pm$ 0.02	0.82 $\pm$ 0.01

## **Discussion**

### **Dry to Fresh Weight Ratios**

With increasing irradiance level, leaves of the freshwater plants had higher DW: FW ratios. As discussed in *Chapter 2*, under salt stress *S. pectinata* again developed in an increased leaf dry to fresh weight ratio, and salt-treatment related differences were much larger than those associated with growth irradiance. This large salt effect is likely due to an accumulation of organic osmo-regulators (Läuchli and Grattan 2007, Munns and Tester 2008, Janda et al. 2016, Acosta-Motos et al. 2017) and/or salt sequestration. Upon exposure to the first salinity level at 6 ppt, increase in DW: FW ratio in comparison to the corresponding controls was more rapid with increasing irradiances. As net photosynthesis was higher with increasing irradiance levels (Figure 2), high irradiance plants potentially accumulated organic osmo-regulators at a faster rate than low irradiance plants and high irradiance, therefore, may allow plants to acclimate to salinity more quickly.



### ***Chlorophyll-a Content and Chl-a standardised Photosynthesis ( $PS_{Chl-a}$ )***

In control plants, Chl-a/FW decreased with increasing irradiance levels, but light saturated  $PS_{Chl-a}$  was always higher for the HL plants than the LL plants (Figure 4). In the photosynthetic light harvesting complex (LHC), chlorophyll-a is a fundamental pigment that harvests photon energy directly or transfers excitation energy from other pigments in LHC (chlorophyll-b and carotenoids) for use in photochemistry (Falkowski and Raven 2007a). At high irradiance, plants and algae tend to decrease light harvesting chlorophyll of both photosystem II (LHCII) and of photosystem I (LHCI), allowing a greater ratio of functional proteins and enzymes for electron transport and carbon fixing to increase maximum photosynthetic rate per unit chlorophyll (Anderson et al. 1995, Walters 2005, Moejes et al. 2017).

Compared to the corresponding control plants, salinity treatments increased Chl-a/FW at the ML and LL. Hootsmans and Vermaat (1994) reported lowered chlorophyll concentrations in *S. pectinata* during vigorous growth, due to the synthesis of chlorophylls not keeping up with fast leaf production. Such a disconnection between leaf production and chlorophyll synthesis may explain the increased Chl-a/FW in the salinity treatments. Elsewhere it was shown that leaf production rates decrease with increasing salinities (Chapter 3), the increases in Chl-a/FW might result from a greater constraint on leaf production than on chlorophyll synthesis. Another plausible reason is a more compacted mesophyll structure in leaves in the salinity plants. (Leaf cell size was smaller in 12 ppt plants than in control plants visually under microscopy). Leaves of *S. pectinata* have a homogeneous mesophyll structure (Ronzhina et al. 2004) and chloroplasts are presented in this mesophyll structure (Acosta-Motos et al. 2017). Also, van den Brink and van der Velde (1993) observed salinity reduced cell expansion and photosynthetic leaf area in *Potamogeton lucens* L., *Potamogeton perfoliatus* L., and *Potamogeton nodosus*. Reduced cell expansion or more compacted mesophyll structure likely results in increase in Chl-a/ FW at ML and LL.

Interestingly, Chl-a/FW for the HL plants only had little increase and this is likely due to the effect of salinity on  $PS_{Chl-a}$ . At the high irradiance level, 12 and 18 ppt salinity treatments reduced the light-saturated  $PS_{Chl-a}$  (Figure 4), indicating an ultimate reduction in the activity

of carbon reactions mediated by the Calvin – Benson cycle (Falkowski and Raven 2007b) by the 12 and 18 ppt treatments. Reduced Calvin-Benson cycle activity increases the proportion of reduced plastoquinone pool between photosystem II and photosystem I, therefore less excitation energy is channelled through electron transport chain, which leads to over excitation in the photosynthetic antennae that is equivalent to high light stress (Anderson et al. 1995, Wilson et al. 2006, Huner et al. 2012). The long-term acclimation to high-light stress is to reduce light absorption by reducing the physical size of photosynthetic antennae (Huner et al. 2012), to an extent which is equivalent to reducing chlorophyll-a content.

### ***Interaction of High Irradiance Acclimation and High Salinity on Light Saturated $PS_{Chl-a}$***

There is a clear interaction of high irradiance acclimation and high salinity on leaf photosynthesis, as substantiated by that light-saturated  $PS_{Chl-a}$  was reduced at 12 and 18 ppt for HL acclimated leaves, at 18 ppt for ML acclimated leaves, but not for LL acclimated leaves nor any plants at 6 ppt. As discussed above, there is an argument that reduced downstream utilisation of photochemical energy mimics increased irradiance stress, and this may in part explain this observation. A further possible reason for the constrained  $PS_{Chl-a}$ , therefore constrained Calvin-Benson activity (Falkowski and Raven 2007b), might be limited by carbon supply. *S. pectinata* uses bicarbonate from water for photosynthesis (Kantrud 1990). Use of bicarbonate is an active uptake process (Madsen and Maberly 2003) and in aquatic angiosperms, is associated with  $HCO_3^-$  dehydration in an acidified zone that is created by protons from the cytosol being pumped outside the plasma membrane during photosynthesis (Price et al. 1985). Hellblom and Axelsson (2003) have shown that bicarbonate utilisation and osmoregulation in *Ruppia cirrhosa* use the same energy source (ATP via  $H^+$ -ATPases in the plasmalemma) and *R.cirrhosa* accumulates proline as an osmoregulator and blackens its leaves during exposure to intolerable high salinities (Adams and Bate 1994). This is similar to symptoms observed in leaves of *S. pectinata* in salinity stress (Chapter 3). The interaction of high irradiance acclimation and high salinity could

possibly be explained by an increased energy requirement for bicarbonate acquisition activity in completion with enhanced energy requirement for osmoregulation.

Reduced photochemical ability to cope with high irradiance in salinity treatments have been reported for other photosynthetic organisms: lower saturating irradiance for isolated mesophyll cell of cowpea leaves (Plaut et al. 1989), reductions in maximum  $PS_{Chl-a}$  for cyanobacteria *Spirulina platensis* (Vonshak et al. 1996), and reduced photosystem II energy conversion efficiency for isolated chloroplast from Barley and Sorghum (Sharma and Hall 1991), and for wheat *Triticum aestivum* L. (Muranaka et al. 2002). The lowered saturating irradiance for chloroplasts of cowpea was due to  $Na^+$  induced damage to the photosynthetic apparatus that only happened after a threshold salinity level was reached (Plaut et al. 1989). For *S. platensis*, the reduced maximum photosynthetic rates by the interaction of salinity stress and high irradiance was due to limited protein synthetic ability due to ionic toxicity of  $Na^+$  and  $Cl^-$  (Reed et al. 1986), resulting in constrained turnover of the D1 protein essential for photo-inhibition recovery (Vonshak et al. 1996). *S. platensis* also showed significant reductions in maximum quantum yield, suggesting photosystem damage (Vonshak et al. 1996). For *T. aestivum*, leaves had lower maximum quantum yield with increasing irradiance level in NaCl treatment, and the extent of impairment in photosystem II was linearly related to the leaf  $Na^+$  content (Muranaka et al. 2002).

For those organisms, the salinity-induced reductions in photosynthesis under high irradiance were essentially due to impairment of the photosynthetic apparatus associated with ionic toxicity (Plaut et al. 1989, Vonshak et al. 1996, Muranaka et al. 2002). Although the photosynthesis in *S. pectinata* in this study was constrained by salinity at high irradiance level, the photosynthetic apparatus was unlikely to be impaired. Photosystem II in *S. pectinata* seems to be fully functional and protected, as substantiated by little difference in the maximum quantum yield (Figure 8) and no reductions in light utilisation efficiency when irradiance was not saturating (Figure 4) (Falkowski and Raven 2007a). As discussed in Chapter 3, newly produced leaves of *S. pectinata* would likely sequester  $Na^+$  in vacuoles until a threshold level, before which photosystems were protected from ionic toxicity. Constraint in light-saturated  $PS_{Chl-a}$  is unlikely due to damage of photosynthetic apparatus, and more likely results from constrained procedures involved in the Calvin-Benson activity (e.g. bicarbonate acquisition) in new fully expanded leaves of *S. pectinata*.

### ***Total Carotenoid Concentrations, Chl-a: Car Ratios, and Non-Photochemical Quenching (NPQ)***

For the control plants, the total carotenoids concentration was higher at HL than at ML and LL (Figure 5). Carotenoids are found to protect photosynthetic antennae and membranes from light-dependent oxidative damage, by scavenging reactive oxidative species generated from chlorophyll triplets, and by dissipating excessive light energy as heat (Ashraf and Harris 2013). The leaves of the HL plants, with growth irradiance ( $340 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) exceeding the  $E_k$  ( $100\text{-}200 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) (Figure 2), likely need to dissipate more light energy from the photosynthetic apparatus than leaves of the ML and LL plants, the growth irradiance for which was either similar to or smaller than their  $E_k$  (Figure 2). This is supported by the fact that *S. pectinata* at LL showed a limited ability for non-photochemical quenching compared to ML and HL plants (Figure 7). The photo-acclimation in leaves of control plants resulted in decreasing Chl-a: Car ratios (Figure 6), suggesting better protection against high light stress (Blindow et al. 2003, Gerhardt et al. 2009) with increasing irradiance. The Chl-a: Caro ratio was further reduced in 12 and 18 ppt treatments at HL, and in 18 ppt treatment at ML (Figure 6), where coincidentally in the same treatments reductions in light-saturated  $\text{PS}_{\text{Chl-a}}$  were observed. The lowered Chl-a: Car ratio in these salinity treatments are likely due to adjustments of LHC to higher light stress, corresponding to the constrained Calvin-Benson cycle activity as indicated by reduced light-saturated  $\text{PS}_{\text{Chl-a}}$  (Anderson et al. 1995, Huner et al. 2012, Moejes et al. 2017). This argument is further supported in our study by that enhanced NPQ was observed in 12 ppt treatments at HL and 18 ppt treatment at ML, where constrained light-saturated  $\text{PS}_{\text{Chl-a}}$  and reduced Chl-a: Car ratios occurred.

Although NPQ was not statistically larger in 18 ppt treatment at HL compared to the corresponding control (Figure 7), there was an unexpected increase in NPQ in the controls at the third sampling compared the first and second. This unexpected increase might obscure the phenomena of enhanced the NPQ in 18 ppt treatment at HL. Therefore, it is concluded that in response to constrained light-saturated  $\text{PS}_{\text{Chl-a}}$  by the interaction of acclimation to high irradiance and high salinity, adjustment in photosynthetic antennae resulted in lower Chl-a: Car ratios and greater non-photochemical quenching ability.

## ***Ecological Implications***

The leaf photosynthesis (per leaf fresh weight) was positively correlated with irradiance in this study (Figure 2), suggesting that *S. pectinata* in Te Waihora (Lake Ellesmere) would have higher photosynthetic rate per unit photosynthetic leaf area by positioning its leaves towards the water surface as shoots grow taller in the turbid water column, where light attenuates exponentially with depth. The increase in Chl-a/FW in salinity treatments for the ML and LL plants suggests a higher capacity to harvest light per unit photosynthetic leaf area than control plants. Given the lack of an effect of salinity on the slope of the light-limited portions of the Chl-a specific P vs I curves, this implies that salt-acclimated plants may have a higher light harvesting capacity per unit photosynthetic leaf area than controls in light stressing conditions. This must be offset, however, by reduced photosynthetic leaf area due to that leaf growth is slower and senescence rate is faster in salt treatments (Chapter 3). Thus, overall salinity stress does not necessarily result in a greater light harvesting capacity for the plant. Indeed, slow-growing, rapidly-senescing, salt-acclimated leaves appear to be much less efficient at developing a photosynthetic canopy than those growing under freshwater conditions (Chapter 3). Once the plant extends its leaves close to the water surface, leaf photosynthesis starts to be stressed by salinity ( $\geq 12$  ppt) because of the interaction of acclimation to high irradiance and salinity.

## **Conclusions**

- 1). In *Stuckenia pectinata*, Chl-a per unit FW decreased with increasing irradiance levels, and increased with elevated salinity at the medium and the low irradiance but not at the high irradiance. Light-saturated  $PS_{Chl-a}$  was constrained by the interaction of acclimation to high irradiance and salinity, and was accompanied by reduced Chl-a/Car ratios and higher non-photochemical quenching ability.
- 2). Net photosynthesis per photosynthetic leaf area increased with increasing irradiance levels. Salinity  $\geq 12$  ppt constrained leaf photosynthesis at high irradiance, whereas salinity had no effect on leaf photosynthesis at medium and low irradiance. This interaction

between salt stress and photophysiology reduces the ability of the plant to optimise performance at high irradiance.

(3). In the context of Te Waihora (Lake Ellesmere), this interaction effect will constrain the ability of surface-reaching, high light plant tissues to drive growth and replenish carbon resources when salinity is high ( $\geq 12$  ppt), whereas salinity has no effects on photosynthesis in the light-limited, lower parts of the water column.

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## Chapter 5: Construction of a Habitat Template and its Verification

Re-establishment of a submerged macrophyte bed, with *Stuckenia pectinata* as a key species in Te Waihora (Lake Ellesmere), is challenged by high turbidity and fluctuating salinities. To define a suitable habitat template in the lake, to allow *S. pectinata* to grow from a sprouting tuber into a surface-reaching shoot (a single growth stage), here I construct a habitat template with respect to light conditions in a turbid water column and salinity as the two limiting factors. The light condition in the turbid water column is related to incident irradiance at the water surface, light attenuation through water column and water level. In order to determine the ranges of these key environmental factors in the habitat template, performance of *S. pectinata* was experimentally studied in low-light conditions, in different salinity exposure scenarios, and at different irradiance levels interacting with various salinity levels.

### Summarising the performance of *S. pectinata* in the experiments and its ecological implications in Te Waihora (Lake Ellesmere)

The greenhouse shading experiment showed that *S. pectinata* under 70% shading had longer stems and leaves, and reach the water surface earlier than the non-shaded plants (*Chapter 2*). These low-light morphological acclimations in *S. pectinata* allowed it to increase the photosynthetic leaf area for light harvesting, and to reach the well-illuminated upper water column sooner. Investigation in leaves acclimated to low versus high light conditions in the lake and laboratory both showed that low-light leaves had higher chlorophyll content and increased photosynthetic rate per dry weight in low light, likely due to enhanced light harvesting (*Chapter 2*). Once the plant reaches the water surface and leaves begin to float, the plant escapes low light stress as a result of turbidity. Photosynthesis-irradiance curves and pigment analysis in leaves acclimated to high irradiance (HL) versus low irradiance (LL) demonstrated that although chlorophyll content was lower in HL leaves, photosynthetic rate per unit chlorophyll-a was higher (indicating higher efficiency of light utilisation), resulting in a higher leaf photosynthetic rate per fresh weight (leaf photosynthetic area) (*Chapter 4*). In addition, plants under 70% shading had shorter leaf longevity than the non-shaded plants

(Chapter 2), early senescence of low-light leaves in the lower part of the water column could redirect resources allocated to the dying leaves to growth of other plant parts (Lim et al. 2007), such as stems and new leaves that are closer to the water surface. To summarise, low-light induced morphological and physiological acclimations in *S. pectinata* would enhance light interception and efficiency of harvesting low light in the turbid water column, accelerate stem elongation, and mobilise resources in old leaves to extend new leaves to the water surface sooner, where light utilisation becomes more efficient.

In addition to turbidity, salinity synergistically stresses the growth of *S. pectinata* in Te Waihora (Lake Ellesmere). Laboratory experiments on growth performance of *S. pectinata* under salinity stress demonstrated reduced leaf production rate with increasing salinities, salt-induced leaf senescence, and reduced leaf length at 12 ppt (Chapter 3). All of these contribute to less photosynthetic leaf area, while increase in leaf photosynthetic area is an advantage of low-light induced morphological acclimation. Slower leaf production rate likely delays the emerging shoots of *S. pectinata* to reach the well-illuminated upper water column, therefore, increases the time of being submerged (low-light stressed). The experiment investigating the effect of salinity on leaf photosynthesis at different irradiance demonstrated that salinity had no effects on leaf photosynthesis at low irradiance levels, but salinity ( $\geq 12$  ppt) reduced leaf photosynthesis (due to reduced light utilisation efficiency) at high irradiance (Chapter 4). This suggests when leaves of *S. pectinata* extends to near or at the water surface, where light is not limiting and light utilisation becomes more efficient for leaves compared to leaves in the water column, photosynthesis would be constrained when lake water salinity fluctuates to 12 ppt and above. To summarise, the overall plant photosynthesis capacity of *S. pectinata* is constrained by salinity stress, via reductions in leaf photosynthetic area morphologically and reductions in photosynthesis of leaves near the water surface physiologically.

Most of the time salinity in the lake fluctuates between 5 and 10 ppt (Gerbeaux 1989). Salinity can increase to 20 ppt occasionally. The increase by more than 20 ppt is associated with lake opening more than 50 days, and it takes at least 3 months for salinity over 20 ppt to return to normal levels (5 to 10 ppt) (Gerbeaux 1989). Under such circumstance, established shoots (aboveground biomass) would likely die completely. The resilience experiment demonstrated that after 7 days in 20 ppt plants can re-generate shoots from the

belowground parts after returning to freshwater (*Chapter 3*). It remains unknown that whether *S. pectinata* could regenerate after 3 months' exposure to a lethal salinity level. But with the current water management regime, an increase in salinity by 20 ppt or more is a rare event.

## Construction of a habitat template

The habitat template integrates incident irradiance, light attenuation, water level and salinity as the key environmental factors, the first three of which are interrelated. For easy handling, the incident irradiance and light attenuation coefficient are set to be constant, therefore, the light condition at certain depth is only related to depth of water. The average daily global radiation for the lake during growing seasons (from November to March) is estimated to be  $19.3 \text{ MJ m}^{-2} \text{ d}^{-1}$  (from NIWA Climate Data, 1981-2010), which converts approximately to  $470 \text{ } \mu\text{mol photons m}^{-2} \text{ s}^{-1}$  photosynthetically active radiation (PAR). The average attenuation coefficient is  $9 \text{ m}^{-1}$  for down-welling radiation and scattering is the main reason for light attenuation (Gerbeaux and Ward 1991). In a moderately turbid water (*San Diego harbour*), the backscattering (scattering angle  $> 90^\circ$ ) only constitutes less than 2% of the total amount of scattered light, with 75% scattered light within  $15^\circ$  away from the direction of the incident beam (Kirk 2011). In moderately to highly turbid waters, where particle scattering is dominating at all angles, the shape of normalised volume scattering function (for estimation of scattering coefficients at all angle) is not expected to change if turbidity increases, because it is determined by the intrinsic properties of particles rather than its concentration (Kirk 2011). Therefore, it is reasonable to assume that the backscattering in the highly turbid water of Te Waihora is ignorable. Light intensity at a certain depth could be estimated based on down-well attenuation coefficient ( $9 \text{ m}^{-1}$  on average), depth, and intensity of incident irradiance at the water surface.

Based on the photosynthesis -irradiance curves fitting parameters for leaves of *S. pectinata* in the three low-light scenarios, the light compensation points (LCP), estimated by dividing dark respiration by alpha (Table 3 in *Chapter 2*), is on average  $6 \text{ } \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ . The water depth at which incident PAR ( $470 \text{ } \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ) attenuates (with a coefficient at  $9 \text{ m}^{-1}$ ) to  $6 \text{ } \mu\text{mol photons m}^{-2} \text{ s}^{-1}$  is 0.48 m, defined as light compensation depth.

Theoretically, a healthy leaf of *S. pectinata* could maintain a positive net photosynthesis once it reaches the light compensation depth, before which the growth of shoots use resources from a germinating tuber. Vermaat and Hootsmans (1994a) reported that tubers of a standard size (0.1 to 0.2 g) supported shoot growth to 18 cm tall in darkness at 13 °C after 40 days since germination, whereas at higher temperatures (15 and 22 °C) tubers only supported plant shoots to less than 10 cm and remained dormant. Given this, the maximum colonisation depth is estimated to be 0.66 m (light compensation depth + shoot length in dark = 0.48 m + 0.18 m) for *S. pectinata* starting from a tuber of 0.1 to 0.2 g. If tubers are less than 0.075 g, less resource is available to support initial plant growth (Vermaat and Hootsmans 1994b), therefore a shorter shoot length in dark. Given the incident PAR of 470  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  at the water surface and a light attenuation coefficient of 9  $\text{m}^{-1}$ , *S. pectinata* could grow from a sprouting tuber to a surface reaching plants in areas less than 0.66 m deep in Te Waihora. Increases in light attenuation, unfavourable weather phenomena (e.g. raining and cloudy) and smaller tubers would constrain the plants to even shallower area, and vice versa.

The growth of a sprouting tuber into a shoot of surface reaching canopy requires utilising light energy via photosynthesis under a gradient of irradiances along the turbid water column. Salinity reduces photosynthetic leaf area and salinity at 12 ppt and above limits photosynthesis of leaves at or near the water surface. The photosynthetic capacity of a shoot is reduced under salinity stress. There is chance that a growing shoot is energy constrained and stops growing before the development of a surface-reaching canopy. In the salinity stress experiment (*Chapter 3*), *S. pectinata* in 12 ppt salinity treatment showed no growth in term of leaf number and a continuous decrease in leaf photosynthetic area (as leaf length decreased with time); in 6 ppt treatments plants demonstrated increases in leaf number and no significant reductions in leaf length (*Chapter 3*). Thus, the chance would be much higher for a shoot to stop growing before reach the water surface in 12 ppt than in 6 ppt. For the habitat template, *S. pectinata*, starting from a germinating tuber, is expected to reach the water surface at < 0.6 m water depth and < 6 ppt salinity in Te Waihora.

Although the salinity effect on shoot length was not addressed in the salinity experiments, (van Wijk et al. 1988) reported no reduction in mean shoot lengths at 5 ppt and 10 ppt salinities, but a 25% reduction at 15 ppt for *S. pectinata* originating from brackish habitats.

They also found a 33% reduction in mean shoot length at 5 ppt and 10 ppt for *S. pectinata* of freshwater habitat origins (van Wijk et al. 1988). It is concluded that *S. pectinata* grows best in term of biomass production at salinities close to their original habitat, either in brackish waters or in freshwater (van Wijk et al. 1988). *S. pectinata* sourced from a tributary of Te Waihora (Lake Ellesmere) seems to be a freshwater ecotype, as it showed the best growth in freshwaters compared to all salinity treatments (*Chapter 3*), similar to those of a freshwater origins (van Wijk et al. 1988). Under the assumption that salinity stress induces a 33% reduction in the shoot length of *S. pectinata* in Te Waihora (Lake Ellesmere), the expected shoot length of 18 cm constructed using tuber resources only would be reduced to 12 cm. The light compensation points for leaves of *S. pectinata* in 0 ppt, 6 ppt, and 12 ppt treatments were estimated to be 6, 8, and 5  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  for respectively (estimated by respiration/ $\alpha$ , data from Table 2, *Chapter 3*) and these values are similar. Thus salinity is not expected to affect light compensation depth for leaves of *S. pectinata*. To summarise, salinity would further constrain the establishment of a sprouting tuber of *S. pectinata* to areas less than 0.6 m deep (light compensation depth + shoot length in dark under salinity stress = 0.48 m + 0.12 m).

In brief, the habitat template for establishment of a sprouting tuber of *S. pectinata* in Te Waihora (Lake Ellesmere) defines the water depth to be less than 0.6 m and salinity to be equal to or less than 6 ppt, with the assumptions of a daily incident PAR of 470  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  at the water surface and a light attenuation coefficient of 9  $\text{m}^{-1}$  in the water column. Increases in light attenuation and decreases in daily radiation at the water surface would further reduce the maximum colonisation depth.

### **Verification of the habitat template with growth of *S. pectinata* in Te Waihora (Lake Ellesmere)**

A small population of *S. pectinata* still occurs in a relatively sheltered site (Timber Yard Point) in Te Waihora (Lake Ellesmere). Monthly investigations of this field population from April 2015 till February 2016 were carried out after seeing it by chance in March 2015. In the field plant survey, *S. pectinata* were surveyed along three transects. Each transect starts from the edge of the lake, advances into the lake along with increasing water depth, and stops at a

point beyond which plants disappear. For each transect, the maximum depth of plant occurrence was recorded, and the aboveground biomass of a relatively dense plant patch within a 0.6 m by 0.6 m quadrat was collected and determined for dry matter (drying at 80°C for 24 h). In the field survey, a few environmental parameters were monitored. These include down-welling light attenuation coefficient, salinity, and water temperature. Average lake water level data and lake opening records for the same period was collected from a report (Kirstein 2017). Light compensation depth is calculated using the down-well light attenuation coefficients, the averaged incident PAR of the growing seasons ( $470 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ), and the compensation irradiance for *S. pectinata* ( $6 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ).

The biomass of the field population of *S. pectinata* decreased over time in the mid-autumn and early winter of 2016 (April to June) and died back in winter of the same year (July until September) (Figure 1). Vigorous regrowth of the population started in late spring of 2016 (October till November 2016). Biomass gradually decreased over the subsequent summer period (November 2016 to January 2017) until a complete dieback at the end of summer (February 2017) (Figure 1).

Table 1. Water temperature, and light attenuation coefficient of the field survey

Date	2016 Apr.	2016 May	2016 Jun.	2016 Jul.	2016 Sep.	2016 Oct.	2016 Nov.	2017 Jan.	2017 Feb.
Light Attenuation Coefficient ( $\text{m}^{-1}$ )	9.1	13.7	12.7	8.4	11.9	13.9	7.4	12.1	17.1
Water Temperature ( $^{\circ}\text{C}$ )	17.5	13.1	9.1	8.0	11.7	14.6	19.7	19.0	22

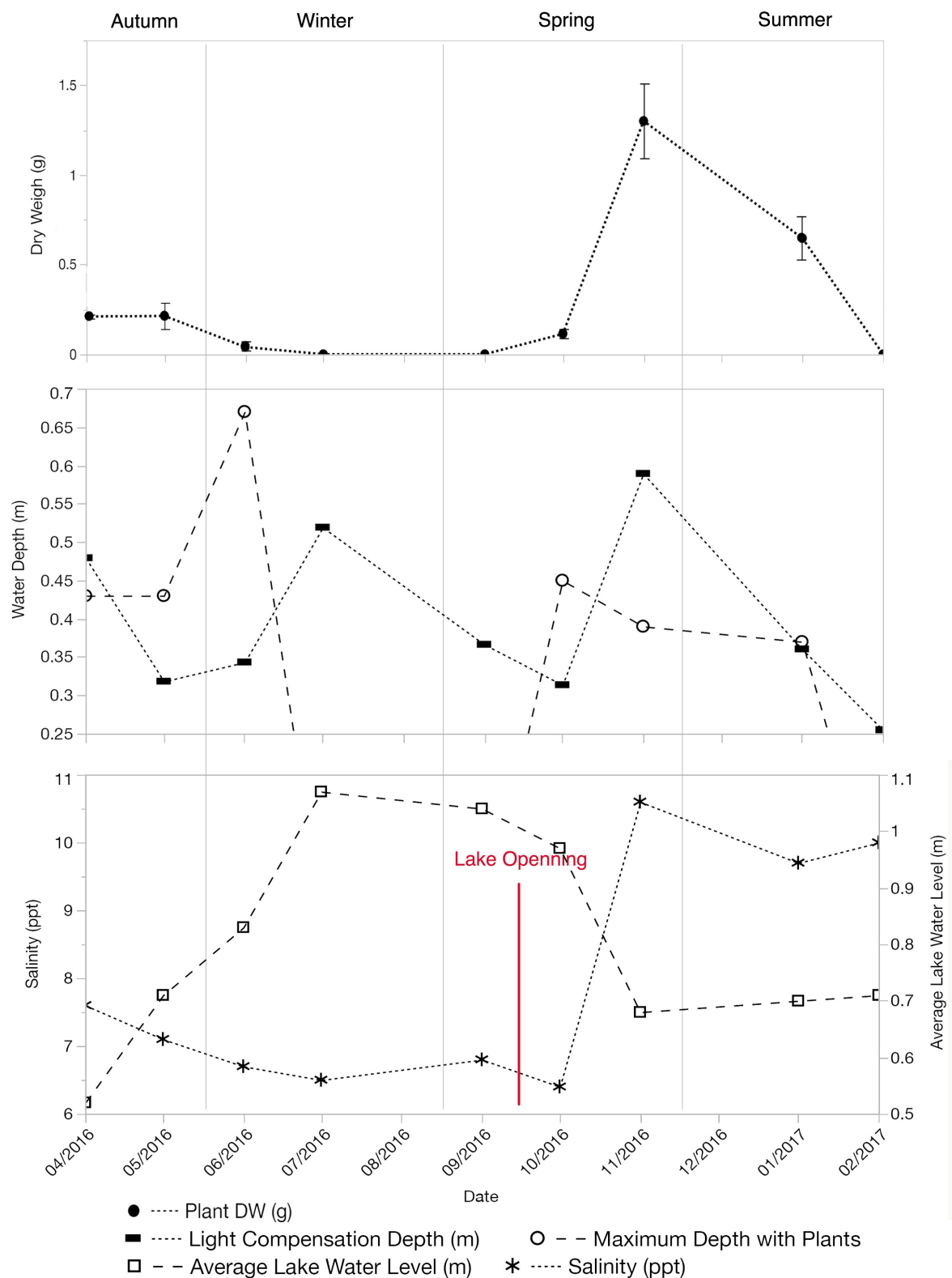


Figure 1. Plant dry matter, estimated light compensation depth, maximum depth with plants, salinity, and lake water level in the monthly field survey of *S. pectinata* from April 2016 to February 2017 (plant dry weight is average of the three transects, and error bar is standard error of the mean)



The recession of *S. pectinata* during the 2016 autumn-winter period coincided with a water level rise from 0.5 to 1.05 m, which increased the maximum depth with plants, to be larger than the light compensation depths (the middle panel in Figure 1). This suggests that parts of the plant were below the light compensation depth and light stressed. Light limitation probably contributes to the dieback of plants in the winter of 2016. The temperature dropped from 17 to 8 ° C. Although the temperature was still above the reported minimum growth temperature (5.5 °C) (van Wijk 1983), it could slow down the growth rate of *S. pectinata* (Spencer 1986) and possibly contributed to the dieback. Salinity during this period was not very volatile and around 7 ppt (bottom panel in Figure 1), similar to the defined safe salinity level of 6 ppt in the habitat template. Most likely, it is the reduction in light condition due to water level raises cause dieback of the plants (Figure 1).

The vigorous growth in the late spring (Oct.-Nov. 2016) coincided with an increasing light compensation depth, water temperature and salinity (Figure 1). Increasing light compensation depth (0.3 to 0.6 m) and temperature (14.6 to 19 °C) all favoured the growth of *S.pectinata*. The vigorous growth suggests *S. pectinata* was tolerable to the gradual increase in salinity (from 6 ppt to 10.5 ppt).

The recession of the plants over the summer of 2017 coincides with a sustained high salinity level at 10 ppt and a low water level (0.7 m). In Nov.2016 and Jan. 2017, the light compensation depth was larger than or similar to the maximum plant depth (middle panel in Figure 1), suggesting plants were not light-limited. The decline of *S. pectinata* in this period could be attributed to the persistently high salinity level around 10 ppt (bottom panel in Figure 1). At the end of summer, very likely the decrease in light compensation depth (0.35 to 0.25 m) and high salinity level (10 ppt) together cause an early dieback of plants. The constructed habitat template of *Stuckenia pectinata* in Te Waihora (Lake Ellesmere) based on salinity and light limitations seems to interpret the dynamic of the field plants well.

### **A habitat template with various light attenuations, salinity and water depth**

A habitat template shall account the salinity fluctuation and variation of light conditions in the lake, the later of which is determined by incident irradiance, light attenuation coefficient,

and water depth. The incident daily irradiance, set constant as  $470 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ , reasonably well indicate the irradiance at the surface water of the lake because it is the averaged daily incident irradiance of the growing seasons over 30 years (1981 - 2010). Field samplings reveal that light attenuation coefficient in 2016 and 2017 varied between 7.4 and  $17.5 \text{ m}^{-1}$  (Table 1). A new habitat template is constructed to estimate the maximum colonisable depth, depending on variable light attenuation coefficient ( $K_d$ ) and salinity (Figure 2). Maximum colonisable depth is estimated based on  $K_d$ , incident PAR, and the light compensation irradiance ( $6 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ). As salinity increases, both leaf production rates (Chapter 3) and plant size (van Wijk et al. 1988) decreases, therefore the maximum colonisable depth is expected to decrease. Once salinity increases to 12 ppt, leaf length and photosynthesis of surface leaves are limited. Therefore, maximum colonisable depth is expected to decrease sharply as salinity approach 12 ppt. At 18 ppt, although plants could survive they are really small in size ( $< 10 \text{ cm}$  tall) (Chapter 4). Maximum colonisable depth diminishes as salinity approach 18 ppt.

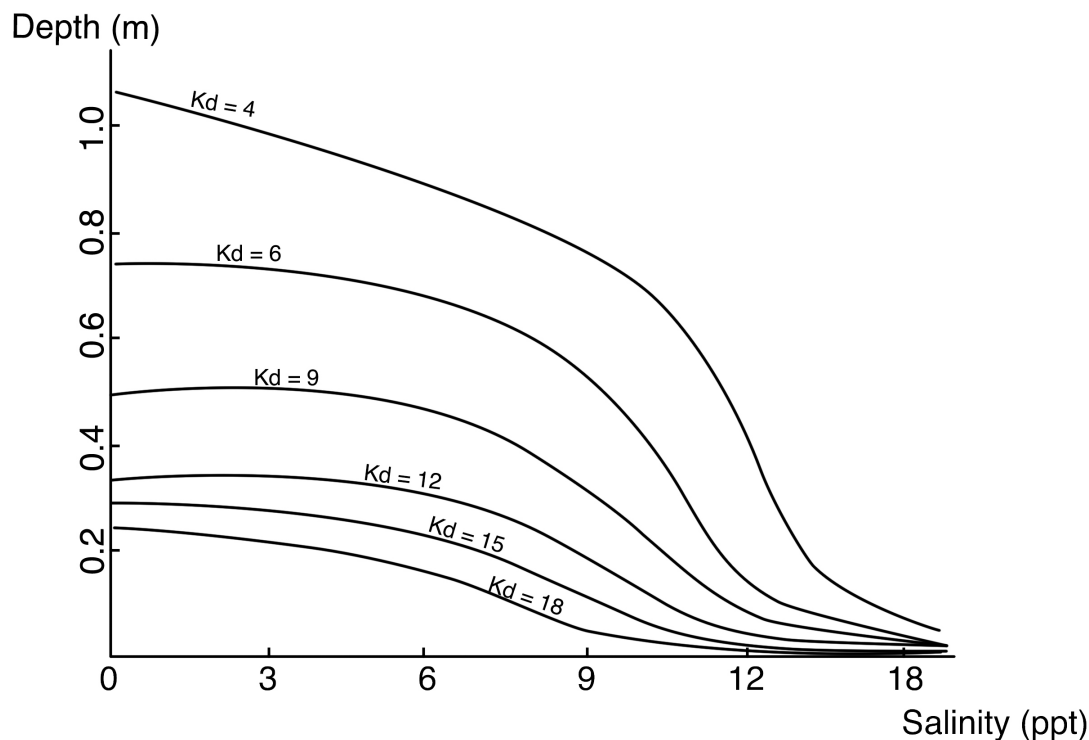


Figure 2. A habitat template of *S. pectinata* in Te Waihora with water depth, light attenuation coefficient ( $K_d$ ), and salinity as limiting factors (the area almost closed by the x-axis, y-axis and the contour line of different  $K_d$  values suggest colonisable conditions)

## Future Research

The initial goal of the habitat template is to define a range for light attenuation, water depth, and salinity to allow *S. pectinata* to grow and persist through consecutive growing seasons. However, the conducted experiments, investigating growth and photosynthesis of *S. pectinata* in response to different light and salinity levels, focused on a single growth stage of the plant. Therefore, the habitat template cannot predict succession of *S. pectinata* through consecutive growing seasons. For *S. pectinata*, belowground parts (rhizomes, roots and tubers) that survived through winter can all contribute to the recovery of the population in the next growing season (van Wijk et al. 1989, Kantrud 1990). In the “Resilience” experiment, *S. pectinata* re-started with belowground biomass after death of the aboveground tissue upon returning to freshwater condition (Chapter 3). The carbohydrate (energy) stored in the belowground biomass could be considered as plant reserve, which could survive winters in unfavourable conditions while aboveground tissues die, and could re-start the plant population when favourable conditions come (van Wijk et al. 1988, van Wijk 1989).

In order to investigate whether *S. pectinata* persist through consecutive growing seasons in the habitat template, additional experiments are needed. First, without salinity stress, two experiments are designed:

1. The growth of submerged macrophyte and its plant reserve in response to a gradient turbidity

In mesocosm conditions, germinating plants (*Stuckenia pectinata*) will grow in a gradient of light attenuations, which is created with shading materials of different light permeability placed at various distance from the plants. Maximum plant biomass and plant reserves are to be estimated. Potential hypothesis is low light attenuation (less than B, Figure 3) does not affect plant biomass and reserve; intermediate attenuation (between B and A, Figure 3) induces plant acclimation to maintain plant biomass and reserve; at high attenuation (larger than A, Figure 3) plant acclimation fail to maintain biomass and reserve.

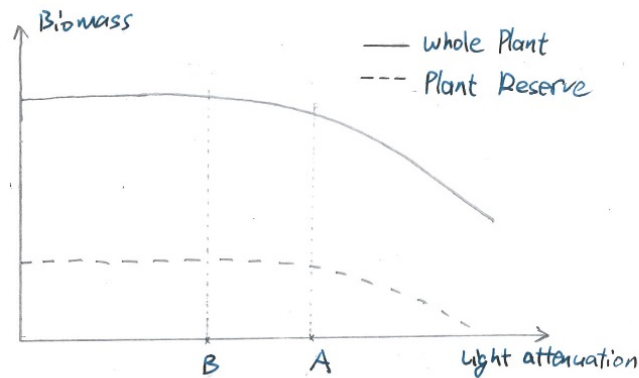


Figure 3. Potential effects of light attenuation on maximum plant biomass and reserve accrual

2. The maximum biomass and plant reserve accrual of *S. pectinata* with different initial plant reserve in low, medium and high light attenuations

In mesocosm conditions, *S. pectinata* with a gradient plant reserves start to grow at high, intermediate and low light attenuation levels, the maximum biomass and plant reserve (of the new growing season) are estimated. Potential hypothesis is increase in initial plant reserve increases maximum biomass and plant reserves in the new growing season (Figure 4). In low and intermediate light attenuation, the maximum biomass and reserves increases with initial plant reserve until environmental carrying capacity (left panel in Figure 4). In high light attenuation, maximum biomass and plant reserve cannot meet the environmental carrying capacity (right panel in Figure 4).



Figure 4. Potential effects of plant reserve on maximum plant biomass and reserve of the next growing season (left: in low and intermediate attenuation; right panel: in high attenuation)

Then the effects of salinity on the relationship between initial plant reserve and maximum biomass accrual, new plant reserve of the new growing season are to be investigated. After

these efforts, the habitat template may predict whether *S. pectinata* persist through consecutive growing seasons under the dual stress of salinity and turbidity.

The constructed habitat template could only be viewed as a basic model to understand the ecology of *S. pectinata* in Te Waihora. Reality is much more complicated to interpret than the simplified habitat template. A limnic ecosystem involves other important interactions: seasonal variation in irradiance and temperature, predator – prey interactions among trophic levels, competition of macrophyte with other primary producers (periphyton and phytoplankton), sediment-water column nutrient convections, and hydrology related external nutrient loading retention. If all these factors could be clearly investigated and integrated into the current habitat template, the habitat template would evolve into an ecological niche for *S. pectinata*.

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